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# THE HAEMOCYANIN OF LIMULUS POLYPHEMUS.

BY C. L. ALSBERG AND E. D. CLARK.

(From the U. S. Bureau of Fisheries Laboratory at Woods Hole, Mass., and the Department of Biological Chemistry of the Harvard Medical School.)

(Received for publication, April 12, 1910.)

The question whether homologous proteins are identical in different animals is one of considerable general interest. It has attracted much attention, and much care has been devoted to it by Osborne<sup>1</sup>. Abderhalden and Schittenhelm<sup>2</sup> have also published upon it. The former compared vegetable proteins, the latter different caseins. Hæmocyanin offers a particularly favorable material for such a study, since, while it is supposed to have the same functions everywhere, it occurs in organisms less closely related than the different species of mammals or the different species of legumes. Henze<sup>3</sup> has made a very complete study of the hæmocyanin of the octopus (*Octopus vulgaris*). He has succeeded in crystallizing it by the original method of Hofmeister as well as by the Hopkins-Pinkus modification, and he has determined a good many of the amino-acids yielded on hydrolysis. We studied the hæmocyanin of *Limulus* and compared our results with those of Henze upon *Octopus*. *Limulus* offers exceptionally good material for a study of this kind, since in the early summer it is very abundant about Woods Hole, and a large female may yield as much as 400 cc. of blood.

The blood was obtained by making an incision in the back at the joint between the head and the abdominal piece. The yield

<sup>1</sup> Osborne and Clapp: *Amer. Journ. of Physiol.*, xx, p. 494; Osborne and Heyl: *Ibid.*, xxii, p. 423.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, xxvii, p. 458.

<sup>3</sup> M. Henze: Zur Kenntniss der Hæmocyanins, *Zeitschr. f. physiol. Chem.*, xxxiii, p. 370. Zur Kenntniss der Hæmocyanins, II. Mitteilung, *Ibid.*, xliii p. 290.

of blood may be increased by doubling up and straightening out the animal at this joint, like opening and closing a bellows, thus squeezing out nearly all of the blood. The blood was allowed to clot, and when the clot had contracted the serum was strained through a cloth. It was then placed in the ice-box for twelve to twenty-four hours in order that the floccules, which we have described in a previous paper<sup>1</sup> (and which Loeb<sup>2</sup> before us has shown simulate a second coagulation), might settle out. These were filtered off. They contain a most interesting protein, upon which we hope to report before long. From the clear serum the hæmocyanin was prepared in one of two ways:

(a) It was dialyzed until all the hæmocyanin was precipitated. The precipitate was then filtered off; dissolved in 5 per cent sodium chloride and fractionated with ammonium sulphate. We think this is not a very desirable method, for the hæmocyanin does not always precipitate completely. We do not quite understand the conditions for the complete precipitation of hæmocyanin by dialysis, but we are under the impression that too lengthy dialysis causes some of it to go into solution. Moreover, we suspect, as we will explain later, that some of the copper may be lost in the process.

(b) The serum was fractionated with ammonium sulphate. We believe this method to be preferable. As the serum was very alkaline it was neutralized carefully with very weak acetic acid, not stronger than .05 per cent, which must be added drop by drop, stirring constantly. If the acid is added too rapidly, or in excess, the hæmocyanin is decomposed, the protein part precipitating and more or less copper remaining in solution. At no time must the serum be allowed to become even faintly acid. It is best to cease adding acid when the reaction is still faintly alkaline. Under these conditions the first protein precipitation appeared when 3.3 parts of saturated ammonium sulphate were contained in a total volume of 10 cc. At this concentration the amount precipitated was slight. It was bluish, showing that it contained hæmocyanin. At a concentration of 4.5 cc. of ammonium sulphate in 10 cc. practically all the hæmocyanin was precipitated. Traces still came

<sup>1</sup> This *Journal*, v, p. 323.

<sup>2</sup> *Beitr. z. chem. Physiol. u. Pathol.*, v, p. 194.



down at a concentration of 4.7 cc. in 10 cc. Further addition caused no precipitation until a concentration of 5.5 cc. in 10 cc. was reached. This precipitate was not very voluminous. It was white, and therefore not hæmocyanin. A last, scanty precipitate was brought down by complete saturation with ammonium sulphate.

All in all, there is very little protein in the blood except hæmocyanin. Indeed we believe, though we have not yet demonstrated it as a fact, that all the protein present except the hæmocyanin is derived from the disintegrating cells of the clot. We base this view on the observation that the serum collected before the clot has begun to contract much is poorer in these proteins than that obtained after full contraction of the clot. We did not pursue this line of investigation further because it ought to be carried out on animals before spawning, when they are in the best condition. Our animals were used later, sometimes after having been kept in a floating car in the harbor several weeks.

We therefore precipitated the serum with 80 cc. of saturated ammonium sulphate solution for each 100 cc. of serum. The crude hæmocyanin thus obtained was filtered off, dissolved in water, and reprecipitated with ammonium sulphate. The necessary ammonium sulphate was added slowly until a concentration of about 3.4 to 3.5 in 10 cc. was attained. The precipitate thus obtained was filtered off and rejected. Then more ammonium sulphate solution was added to the filtrate until a saturation of about 4.4 cc. in 10 cc. was reached. The precipitate thus formed was filtered off and preserved. In this way the material precipitated at the lower and upper limits was rejected, the presumption being that contaminating material was thereby removed. There is of course no guaranty that by this procedure protein with the same precipitation limits as hæmocyanin is removed. That no such protein occurs in the serum we are not prepared to say. We have encountered no evidence of its existence. The hæmocyanin precipitated was redissolved and reprecipitated twice more in this fashion. After the first precipitation the limits of salt concentration were a little higher than they were in the original serum, possibly because the serum itself has about the concentration of salts found in sea-water. After the last precipitation the hæmocyanin was redissolved and precipitated with alcohol, under which it was kept for some time

to coagulate it thoroughly. The alcohol was then decanted off and the coagulum washed free from salt. There was of course the danger that the coagulum might retain traces of the sulphate. If, however, the alcohol were added slowly, with constant stirring, a very light flocculent precipitate was obtained which was easily washed. Moreover, only just enough alcohol to coagulate should be added, so as to keep as much of the sulphate in solution as possible. The sulphate may of course be dialyzed away before adding the alcohol, and thus this source of error may be avoided. However, we feared losing copper (cf. below) more than adsorbing salt. The material thus obtained was analyzed, after having been powdered and dried to constant weight *in vacuo* over sulphuric acid at a temperature of 70°. It gave the following figures:

0.2174 gm. substance Preparation I yielded 0.1405 gm. H<sub>2</sub>O and 0.3891 gm. CO<sub>2</sub>:

H = 7.18 per cent.

C = 48.80 per cent.

0.2094 gm. substance Preparation II yielded 0.1324 gm. H<sub>2</sub>O and 0.3769 gm. CO<sub>2</sub>:

H = 7.02 per cent

C = 49.09 per cent.

0.1950 gm. substance Preparation I yielded 27.30 cc. N at 18° and 766 mm.:

N = 16.30 per cent

0.2221 gm. substance Preparation II yielded 31.02 cc. N at 19° and 760.5 mm.:

N = 16.06 per cent

Sulphur was determined according to Folin. 0.4086 gm. substance Preparation I yielded 0.0465 gm. BaSO<sub>4</sub>:

S = 1.56 per cent

Copper was determined by incinerating in porcelain, and exhausting the ash with nitric acid. A trace of material, probably silica, did not dissolve. The presence of silica in the clot has already been reported by us<sup>1</sup>. The acid solution was evaporated to dryness with a little H<sub>2</sub>SO<sub>4</sub>. The solution taken up in a little very dilute H<sub>2</sub>SO<sub>4</sub> was filtered and the copper determined electrolytically.

0.5115 gm. substance Preparation I yielded 0.0015 gm. Cu:

Cu = 0.29 per cent.

For the check analysis a little more material was taken and a different method used. The material in this case was dried to

<sup>1</sup>*Loc. cit.*

constant weight in the oven at 105°. The ash was exhausted with nitric acid, the nitric replaced with hydrochloric acid, the copper precipitated as the sulphide, the latter collected on filter paper, washed, ignited, converted into the nitrate, and the latter into the oxide by ignition.

0.9960 gm. substance Preparation II yielded 0.0034 gm. CuO:

Cu = 0.0027 gm. = 0.27 per cent

The hæmocyanin obtained by Henze from the octopus had a considerably different composition. The following are his results and ours side by side:

	OCTOPUS. (Average) per cent.	LIMULUS. (Average) per cent.
C.....	53.66	48.94
H.....	7.33	7.10
N.....	16.09	16.18
S.....	0.86	1.56
Cu.....	0.38	0.28
O.....	21.68	25.94

It would seem from this comparison that the two substances are different. The greatest difference is in the sulphur content which is too great to be due to differences in the methods of determination used. The difference in the copper content is also considerable if we assume that our values are correct. We realize fully that to establish them we need more determinations with larger quantities of material. Perhaps it may be found that animals more sluggish than the octopus possess hæmocyanins of lower copper content, and therefore less active oxygen carriers. Certainly their blood as a whole usually contains less copper.<sup>1</sup> The difference in the carbon content of our preparation as compared with that of Henze is also great, and argues for the individuality of this substance.

It is, of course, possible that the difference in our figures is due to the fact that we worked with an impure substance. We do not regard this as probable, although we must admit that we have not the same guaranty of purity that Henze had, for we were quite unable to crystallize this substance as he did his. The Hofmeister method failed us, while in its Hopkins-Pinkus modifi-

<sup>1</sup> Cf. v. Fürth: *Vergleichende chemische Physiologie der niederen Tiere*.

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cation it could not be used at all because of the great sensitiveness of this substance to acids. If to the solution we added a few drops of acetic acid, as Henze did, we got a heavy, curdy precipitate which could not be brought back into solution except by the use of alkali. We tried to obtain this substance in crystalline form, both from the fresh serum as well as from purified hæmocyanin. We are well aware our negative results do not prove that the substance is uncrystallizable. The conditions are not as favorable for its crystallization in *Limulus* as they seem to be in *Octopus*. It is so difficult to prevent larger quantities of the blood from clotting that we were compelled to work with the serum instead of centrifugated blood. The process of clotting as shown by Loeb<sup>1</sup> consists of an agglutination of cells, accompanied by their disintegration. This, as already indicated, we believe to be accompanied by the passage of material from the cells into the serum, which might well interfere with crystallization. Furthermore, Henze<sup>2</sup> states that crystallization goes on well only with fresh blood from healthy animals. While we used fresh blood, we had to lose some time in getting rid of the clot. Moreover, our animals, while apparently in good condition, had been collected and kept in a floating car in the harbor several weeks before they were used. Possibly with animals taken before spawning and used immediately the result might have been different. While these considerations may account for our inability to obtain crystals, we are inclined to believe that the real reason is that *Limulus* hæmocyanin is a globulin.

So much for our quantitative studies, which all point to the individuality of *Octopus* and *Limulus* hæmocyanin. Our qualitative studies offer more evidence for the same conclusion.

Henze states that his hæmocyanin could not be precipitated by dialysis, and questions the results of Halliburton, who reported that it could be so precipitated. Henze does not seem to have taken into consideration that there might be more than one kind of hæmocyanin in nature, and that both he and Halliburton might be right, each for the hæmocyanin with which he worked. As a matter of fact, *Limulus* hæmocyanin may be completely precipitated from serum by dialysis. Solutions of pure hæmocyanin are not as

<sup>1</sup> *Loc. cit.*

<sup>2</sup> *Loc. cit.*, p. 374.

easily completely precipitated, and occasionally behave in an erratic way that we have not yet explained to our own satisfaction, though we think it was probably resolution from too long dialysis. Always, however, the greater part was precipitated. Our hæmocyanin behaved like a globulin, Henze's like an albumen. Our hæmocyanin could be completely precipitated by magnesium sulphate, Henze's could not be. 4.7 cc. saturation with ammonium sulphate completely precipitates it. Henze's is only precipitated by complete saturation. In this respect, too, *Limulus* hæmocyanin behaves like a globulin, *Octopus* hæmocyanin like an albumen. By carbon dioxide our hæmocyanin was only incompletely precipitated, agreeing in this respect with Henze's. It gave the protein precipitation reactions, the xanthoproteic, the biuret, and Millon's reaction. It was precipitated by the salts of the heavy metals.

We are not certain how far the behavior of *Limulus* hæmocyanin toward acids differs from that of *Octopus* hæmocyanin, for we do not quite understand (no doubt our own fault) how the statements of Henze, on pp. 377 and 380 of his first paper, are to be reconciled with one another. We get the general impression from reading his papers that our substance was more sensitive to acid than his. Very slight traces of dilute acid, including acetic, precipitated it as white floccules poor in copper. Further addition of acid did not redissolve it. The copper is in exceedingly loose combination.<sup>1</sup> It may even be completely removed by dialysis if the water used be kept very faintly acid. The dialysis must be very protracted, extending over a number of days. If enough salt be present, the protein will not be precipitated. A cold storage room, kept just above zero, enabled us to carry out this experiment without bacterial action.

The possibility of losing copper in this way kept us from employing dialysis as a step in preparation. We have convinced ourselves that lengthy dialysis sometimes, not always, results in a loss of copper even when, apparently, the solution has remained

<sup>1</sup> One of us has already called attention to the ease with which copper is removed from *Limulus* hæmocyanin (C. L. Alsberg: Beiträge zur Kenntniss der Guajakreaktion. *Arch. f. exp. Path. u. Pharmak.*, Supplementband, "Schmiedeberg Festschrift," 1908, p. 39); and he has also suggested (*ibid.*, pp. 41-42) that different hæmocyanins may differ in their susceptibility to acid.

## 8 Haemocyanin of *Limulus Polyphemus*

neutral. We spoiled considerable material before we realized this possibility. One preparation was dialyzed five days in the cold storage room at a temperature but slightly above zero. It gave us the following figures for copper:

2.7999 gm. substance Preparation III yielded 0.0040 gm. Cu (electrolytic method):

$$\text{Cu} = 0.143 \text{ per cent}$$

To make certain that the figures were correct, a larger quantity of material was used, and the sulphide method employed:

### II

16.434 gm. substance Preparation III yielded 0.0291 gm. CuO:

$$\text{Cu} = 0.138 \text{ per cent}$$

Like Henze, we were unable to obtain any copper compound analogous to hæmatin. We regard our substance as a copper globulin compound, while he regards his as a copper albuminate. We believe they are probably two different proteins, though we are well aware that this can be settled finally only by a quantitative hydrolysis for which we lack at present sufficient material.

### SUMMARY.

The hæmocyanin of *Limulus* differs from that of *Octopus* in percentage composition, in its precipitability by dialysis, by full saturation with magnesium sulphate and by half saturation with ammonium sulphate, in not having been crystallized, and perhaps in being more sensitive to acid. There are therefore different hæmocyanins, and perhaps this fact accounts for the discrepancies in the literature concerning the properties of this substance.

# ON THE PREPARATION OF CYSTIN.

By OTTO FOLIN.

*(From the Biochemical Laboratory of Harvard Medical School, Boston.)*

(Received for publication April 20, 1910.)

Since Mörner's admirable work on the isolation of cystin from among the acid hydrolysis products of the proteins no material improvement has been made on his method for obtaining this interesting amino acid. That method was devised solely for the purpose of obtaining the maximum yield of cystin and therefore without regard to the amount of time and labor involved. For the purpose of obtaining cystin Mörner's method is altogether too long and laborious. Embden<sup>1</sup> came very near finding a very much more convenient method, but through an unfortunate oversight threw away most of the cystin as "melanin" and as a result obtained only a little cystin mixed with much tyrosin. Friedmann<sup>2</sup> adopted Embden's method without correcting the error. He introduced an additional error by using large quantities of boneblack at the stage where the cystin is precipitated. Embden's substitution of a few hours' boiling of the horn or hair with concentrated acid for Mörner's extraordinarily long boiling with weaker acids was, however, a step in advance. In all the methods so far proposed no suitable allowance has been made for the fact that cystin is quickly decomposed by alkalies, even by the weaker alkalies such as sodic carbonate and ammonia, a difficulty which all must have encountered when precipitating cystin from acid solutions by the addition of alkalies, especially when, as is most frequently the case, concentrated caustic soda is used for the purpose.

The method for preparing cystin described in this paper has been used as a laboratory exercise for the past two years by our first

<sup>1</sup> *Zeitschr. f. physiol. Chem.*, xxxii, p. 96, 1901.

<sup>2</sup> *Beitr. z. chem. Physiol. u. Pathol.*, iii, p. 15, 1903.

year medical students and at least 75 per cent of the men have obtained very satisfactory results. The isolation of the cystin is based on its insolubility in acetic acid.

As it is now difficult to obtain genuine hair, while pure wool free from oil is easily obtained at a moderate cost, we use wool almost exclusively as starting material. Wool, like hair, has the great advantage over horn in that it consists of fine fibres and therefore is attacked much more rapidly by hot acids.

From 50 to 500 grams of wool is pushed into a (Jena) flask and concentrated hydrochloric acid (200 c.c. for each 100 grams of wool) is added. In order to get a part of the acid quickly to the bottom of the flask a part of the acid may be put in first, then the wool, and finally the remaining acid. A condenser consisting only of a glass tube 2 to 3 feet long is inserted and the mixture is boiled until the biuret reaction is entirely negative. The wool dissolves in a few minutes and if much cystin is desired more wool and acid can then be introduced. After 3 to 5 hours boiling with moderate quantities of wool the biuret reaction has usually disappeared.

To the hot acid solutions of amino acids so obtained is added at once an excess of solid sodic acetate, *i.e.*, until the congo red reaction for mineral acids is entirely negative. A dark, heavy precipitate containing practically all the cystin is obtained. After a few hours' standing at room temperature the liquid is filtered off and the precipitate is washed with cold water. From the mother liquor diluted with the washwater is usually obtained on long standing a second precipitate consisting chiefly of tyrosin.

The crude cystin is then dissolved in boiling 3-5 per cent hydrochloric acid and the solution is decolorized with good boneblack which should have been previously thoroughly digested with hot, dilute hydrochloric acid and then washed with water in order to remove the calcium phosphate. The hot filtrate from the boneblack should be as clear as water. If it is not perfectly colorless the boneblack treatment should be repeated and if a colorless solution is not then obtained the fault lies with the quality of the boneblack. The last filtrate is heated to boiling and the cystin precipitated by a slow addition of concentrated hot sodic acetate solution.

Large amounts of colorless cystin consisting of typical hexagonal plates can thus be prepared without difficulty and with very little labor.



# EXPERIMENTS RELATING TO THE MODE OF DECOMPOSITION OF TYROSINE AND OF RELATED SUBSTANCES IN THE ANIMAL BODY.

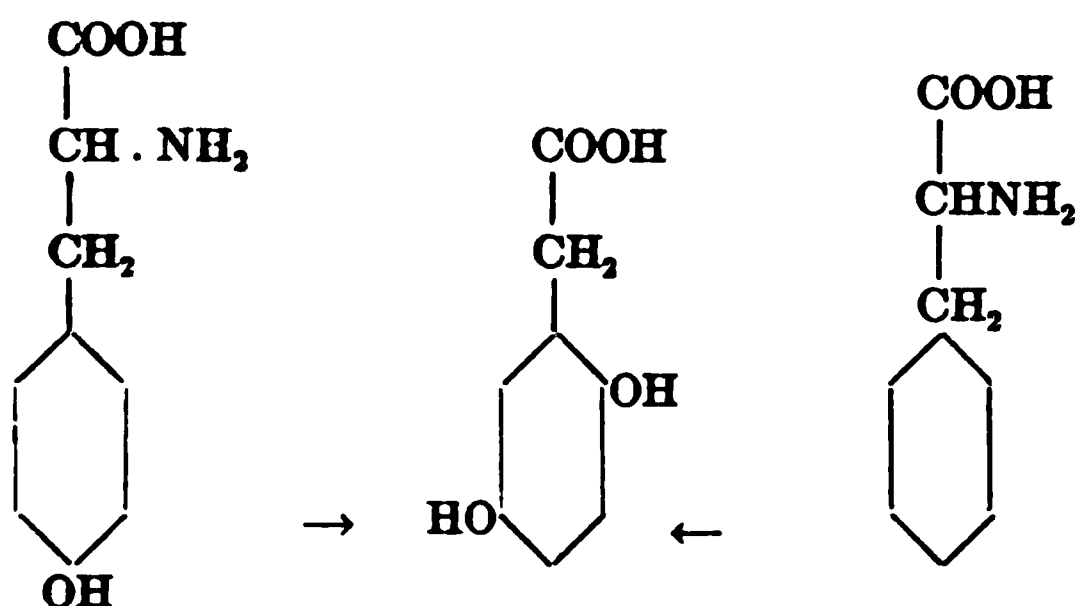
By H. D. DAKIN.

(From the Laboratory of Dr. C. A. Herter, New York.)

(Received for publication, April 13, 1910.)

In spite of the large amount of work directed of recent years towards the solution of the problem of the mode of decomposition in the animal body of the aromatic amino-acids—tyrosine and phenylalanine—we are still without a clear picture of the mechanism of the process which will completely harmonize with the ascertained facts. The object of the following paper is to record the results of some experiments having a bearing upon some of the possible transformations of the amino-acids in question.

In considering the nature of the reactions which tyrosine and phenylalanine are likely to sustain in the animal body, much weight is naturally placed upon the phenomena presented by the condition of alcaptonuria in which, as is well known, the human organism loses its customary capacity of completely oxidizing phenylalanine and tyrosine, but instead converts them into homogentisic acid.



The question at once presents itself: Does homogentisic acid represent an intermediary step in the *normal* catabolism of phenyl-

alanine and tyrosine? This is certainly a difficult question to answer, since it is possible to adduce evidence of fair quality in support of both the affirmative and the negative proposition.

The view that the normal path of catabolism of phenylalanine and tyrosine is through the stage of homogentisic acid was first propounded by Garnier and Voirin,<sup>1</sup> and an excellent account of the reasons for and against the acceptance of their hypothesis is to be found in the Croonian Lectures by A. E. Garrod.<sup>2</sup> Garrod on the whole is inclined to accept the hypothesis of the formation of homogentisic acid as an intermediate product in the normal catabolism of phenylalanine and of tyrosine.

The following paper contains a number of facts which are difficult to harmonize with this view, although they by no means disprove it.

In considering the mechanism of the reactions involved in the conversion of phenylalanine into homogentisic acid, it will be seen that three distinct types of change are requisite. These may be grouped together as follows, although it is not to be assumed that they take place in the order in which they are recorded:

(a) The conversion of a  $\text{CH}_2\text{CH.NH}_2\text{COOH}$  side-chain into a  $\text{CH}_2\text{COOH}$  group. This type of reaction is frequently encountered in both "pure" and biological chemistry. It is a change characteristic of the oxidation of amino-acids with hydrogen peroxide,<sup>3</sup> and other oxidizing agents and is very frequently brought about by the action of micro-organisms (e. g., the formation of *p*-oxyphenylacetic acid from tyrosine).

(b) The introduction of hydroxyl groups into the aromatic nucleus. This change also has many analogies with other reactions taking place in the animal body, and has also been observed to occur when aromatic substances are oxidized with some few oxidizing agents, notably hydrogen peroxide and persulphates.<sup>4</sup>

(c) A molecular rearrangement by which the second hydroxyl group occupies the para-position to the first, whilst the  $\text{CH}_2$  side-

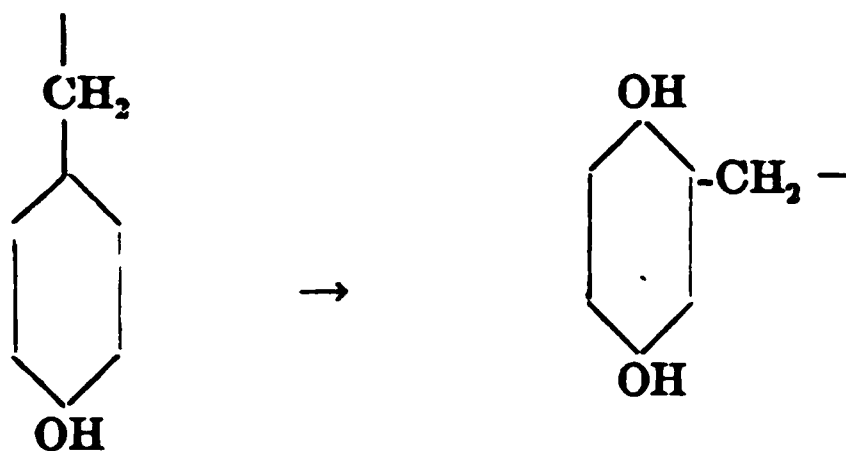
<sup>1</sup> *Archives de physiologie*, iv, p. 225, 1902.

<sup>2</sup> *Inborn Errors of Metabolism*. Oxford Medical Publications, 1909.

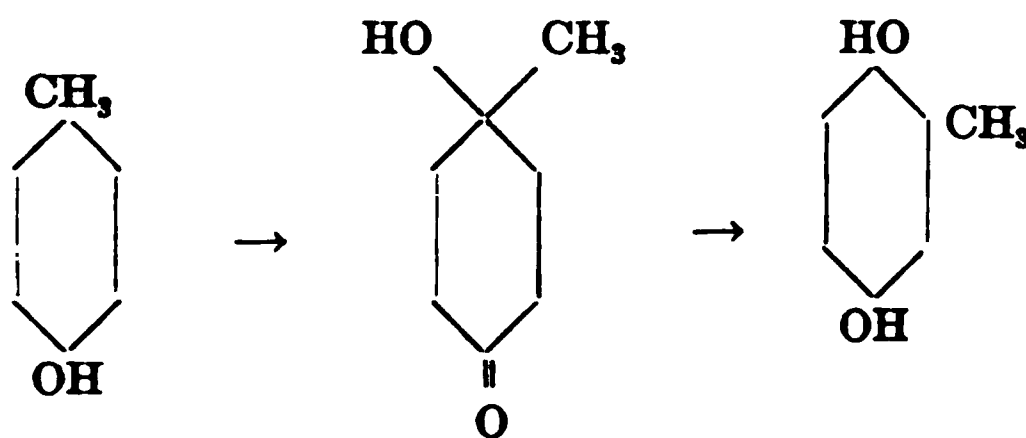
<sup>3</sup> *This Journal*, i, p. 171; ii, p. 63; v, p. 409.

<sup>4</sup> *Ibid*, iii, p. 519.

chain wanders to the ortho-position with respect to the second hydroxyl:



A number of analogies for this type of change are to be found in the investigations of Bamberger,<sup>1</sup> Kumagai and Wolffenstein,<sup>2</sup> Auwers, and others, upon substances belonging to the group of "Quinols." For example, paracresol on oxidation with Caro's acid gives a substance of quinonoid structure which readily undergoes conversion into tolu-hydroquinone:



This last type of reaction is so remarkable that it is very natural to infer that a similar transformation took place in the production of homogentisic acid from tyrosine. The first suggestion of this nature was, I believe, made by E. Meyer,<sup>3</sup> and later more definitely postulated by Friedmann<sup>4</sup> and by Neubauer.<sup>5</sup>

<sup>1</sup> *Ber. d. deutsch. chem. Ges.*, xxxvi, p. 2031.

<sup>2</sup> *Ibid*, xli, p. 297.

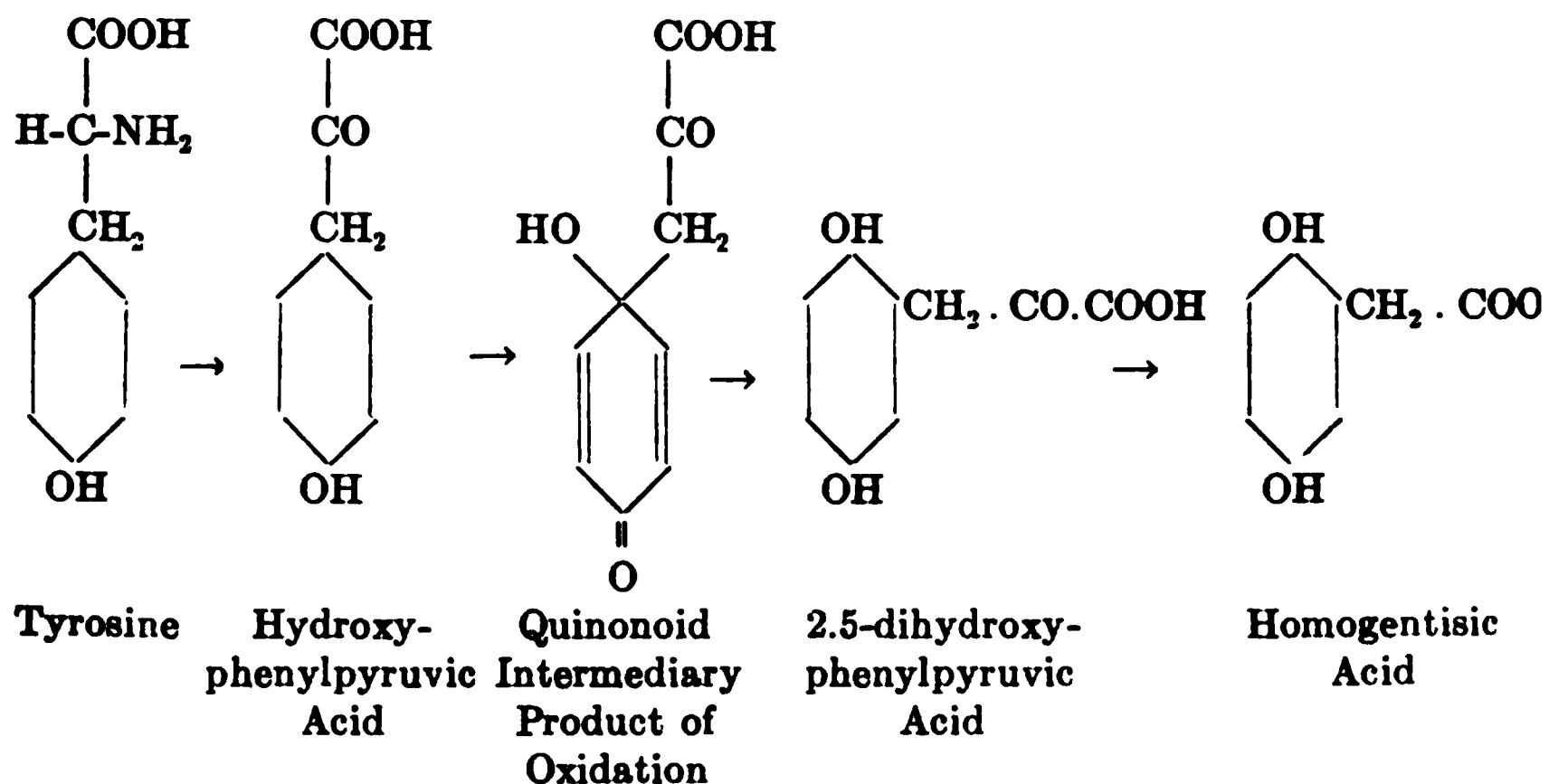
<sup>3</sup> *Deutsches Arch. f. klin. Med.*, lxx, p. 447, 1901.

<sup>4</sup> *Beitr. z. chem. Physiol. u. Path.*, xi, p. 305.

<sup>5</sup> *Deutsches Arch. f. klin. Med.*, xcv, p. 211, 1909.

The writer several years ago made unsuccessful attempts to oxidize tyrosine directly to homogentisic acid by means of potassium persulphate acting under various conditions. It was found practicable to introduce a second hydroxyl group but the entering group in all cases took up a position ortho relative to the original hydroxyl group. In this way catechol derivatives were obtained. It is conceivable that these experiments may have some bearing upon the formation of adrenalin but they throw no light upon the origin of homogentisic acid.

From a chemical standpoint it would appear very probable that a substance of "quinonoid" type is the precursor of homogentisic acid. Neubauer in his suggestive studies upon the origin of homogentisic acid pictures the mechanism of the reaction as follows:



It was considered possible that *evidence might be obtained as to whether homogentisic acid was a normal product of tyrosine catabolism by observing the fate in the body of a single tyrosine derivative in which substitution in the para (OH) group would inhibit the formation of a paraquinonoid derivative.* With this idea in view, the methyl ether of tyrosine (paramethoxyphenylalanine) was synthesized and its behavior in the animal body was studied.

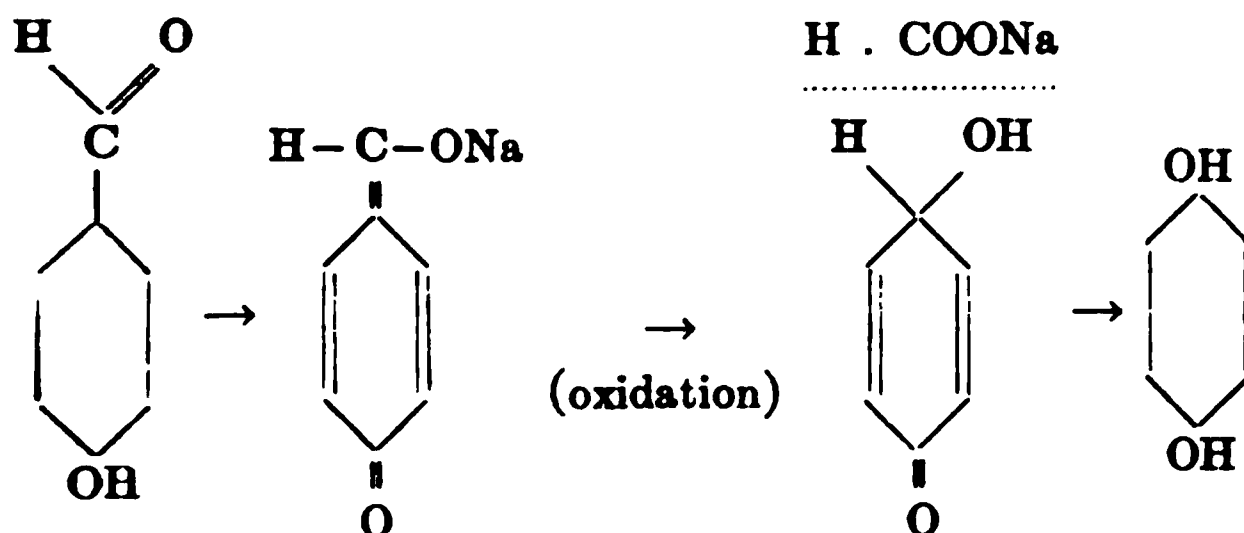


The substance was found to closely resemble tyrosine in both chemical and biological respects. It underwent combustion in the animal body apparently no less easily than tyrosine itself, and, with the exception of a small amount of an acid which we identified as *p*-methoxyphenylacetic acid, no product of its oxidation could be detected. In one experiment 6.0 grams were given to a cat and were well absorbed. No unchanged amino-acid could be detected in the urine, and only 0.4 gram impure methoxyphenylacetic acid was recovered. An administration of a similar amount of racemic

tyrosine to the same animal was followed by the excretion of a small amount of tyrosine in the urine and a notable increase in the oxyphenylacetic acid.

From these results *the inference appears justifiable that the formation of a paraquinonoid substance from tyrosine is not a necessary condition for its combustion.* It might be argued that there is no proof that in the catabolism of paramethoxyphenylalanine the methyl group is not removed with formation of ordinary tyrosine. This appears very improbable, however, for, in the first place, the chemical change involved is a relatively difficult one; in the second place, there is no evidence that such a reaction ever occurs in the animal body,<sup>1</sup> and, thirdly, the methyl group was still intact in the methoxyphenylacetic acid recovered from the urine.

The author has recently described a reaction by which the salts of an ortho- or para-hydroxy aldehyde or ketone may be converted into catechol, quinol or their derivatives by simple oxidation with hydroxygen peroxide.<sup>2</sup> For example, the sodium salt of *p*-hydroxybenzaldehyde when added to hydrogen peroxide is almost quantitatively converted into hydroquinone; formic acid and carbon dioxide also being formed. There is good reason to believe that the reaction takes place between a quinonoid modification of the salt of hydroxy derivatives, since substitution of the hydrogen atom of the phenolic group by an alkyl group inhibits the reaction, and moreover no similar reaction takes place with the meta-hydroxy derivatives of benzaldehyde in which quinonoid rearrangement is unlikely to occur. In the case of *p*-hydroxybenzaldehyde the change may be represented as follows:



<sup>1</sup> Anisic acid (*p*-methoxybenzoic acid) is excreted either unchanged or in combination with glycocholic acid when administered to animals.

<sup>2</sup> *Proc. Chem. Soc.*, 1909. *Amer. Chem. Journ.*, xlii, p. 477.

An inspection of the mechanism of this reaction will show that there is an analogy between this type of change and the formation of homogentisic acid from tyrosine derivatives and since in many cases a close resemblance exists between the oxidations effected with hydrogen peroxide and those occurring in the living organism, it was obviously of interest to investigate the behavior of *o*- and *p*-hydroxybenzaldehyde in the animal body. In no case could any hydroquinone or catechol be detected in the urine when the aldehydes in question were administered to cats or rabbits. The sodium salt of orthohydroxybenzaldehyde was in large measure converted into salicylic acid while the parahydroxybenzaldehyde gave a good yield of both *p*-hydroxybenzoic acid and *p*-hydroxyhippuric acid. No evidence, therefore, was forthcoming from these experiments leading to the belief that any formation of a dihydroxybenzene derivative had occurred in the animal body as the result of the transformation of a quinonoid salt of *o*- or *p*-hydroxybenzaldehyde.

In this connection reference may be made to the recently published investigations of Weevers<sup>1</sup> upon the enzymes present in young shoots of *Salix purpurea*. This author has discovered enzymes capable of forming catechol from salicyl alcohol. It is obvious that this reaction bears a close analogy to the oxidation of salicylic aldehyde to catechol by means of hydrogen peroxide.

To sum up: The results of the foregoing experiments indicate a failure to demonstrate in the normal animal the type of change believed to be necessary for the conversion of tyrosine or phenylalanine into homogentisic acid. The inference that homogentisic acid is not a normal product of catabolism is supported by the following observations, which are, however, admittedly inconclusive: The administration of phenylalanine and tyrosine to animals in such large quantities that much appears unchanged in the urine is not followed by any appearance of homogentisic acid in the urine (cf. following paper). Neither in the case of phenylalanine was any increase of phenolic substances observed, so that at present no evidence of the conversion of phenylalanine into tyrosine such as is pictured by Neubauer, is available. The recent discovery of Jaffe's<sup>2</sup> of the presence of muconic acid in the urine of dogs which

<sup>1</sup> *Proc. K. Akad. Wetensch.*, Amsterdam, xii, p. 193, 1909.

<sup>2</sup> *Zeitschr. f. physiol Chem.*, lxii, p. 58.

have received considerable doses of benzene suggest the possibility that the oxidation of the aromatic nucleus in phenylalanine and tyrosine may not necessarily be preceded by the introduction of (OH) groups into the ring.

The frequent recurrence of the  $\text{CH}_2\text{.CH NH}_2\text{.COOH}$  grouping in the amino-acids of the fatty, cyclic and aromatic series which undergo complete decomposition in the animal body appears very significant. It is of course well known that this group readily undergoes condensation with other amino-acid groupings present in the tissues and it may be that such a condensation is a necessary preliminary to the oxidation of the aromatic nuclei present in tyrosine and phenylalanine. The fact that phenylaminobutyric acid apparently undergoes oxidation in the body with ease and in the case of alcaptonuria is not converted into an alcapton acid (Knoop), is in harmony with this supposition, as are also the observations that substitution of the hydrogen of the amino-group by methyl groups tends to produce substances more resistant to tissue oxidation<sup>1</sup> and that substitution of the  $(\text{CH}_2)$  group by  $(\text{CHOH})$  also changes the course of oxidation. Thus the writer has shown that phenylserine ( $\text{CH}_5\text{.CHOH.CHNH}_2\text{.COOH}$ ) does not undergo oxidation in the tissues along the same lines as phenylalanine but is converted into benzoic acid, which is excreted in the form of hippuric acid.<sup>2</sup>

#### EXPERIMENTAL.

##### *Synthesis of Paramethoxyphenylalanine (Tyrosine methyl ether).*

This substance was obtained from anisic aldehyde by a series of reactions analogous to those taking place in Erlenmeyer and Halsey's<sup>3</sup> synthesis of tyrosine from *p*-hydroxybenzaldehyde.

Anisic aldehyde and hippuric acid were condensed by means of acetic anhydride to form the yellow anhydride ("azlactone")<sup>4</sup> of benzoylamino-paramethoxycinnamic acid, from which the colorless free acid was obtained by dissolving the anhydride in alkali and precipitating with mineral acid. On reducing the latter acid

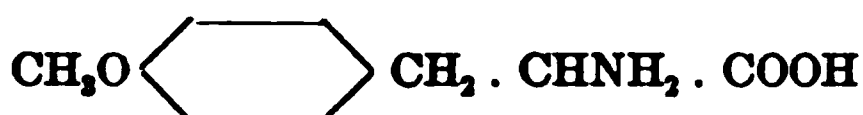
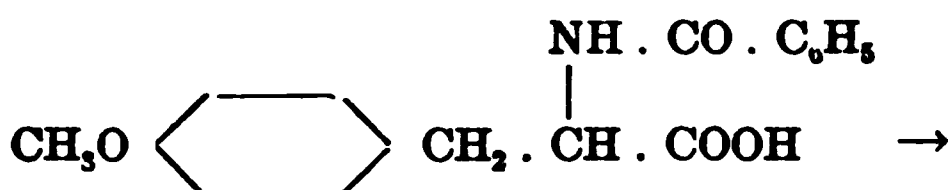
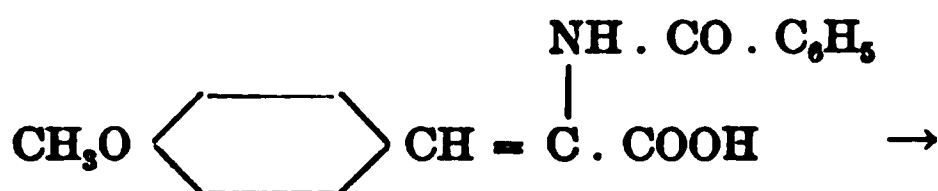
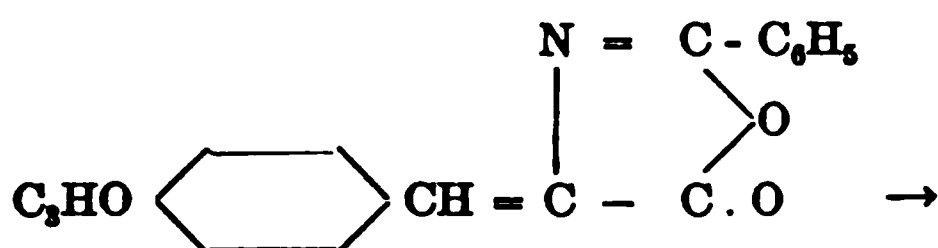
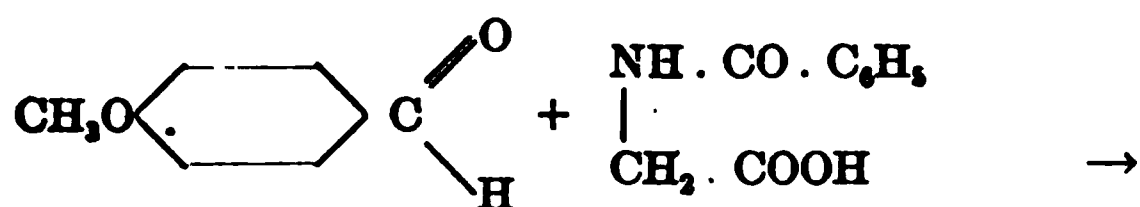
<sup>1</sup> Friedmann: Hofmeister's *Beiträge*, xi, p. 194.

<sup>2</sup> This *Journal*, vi, p. 235.

<sup>3</sup> *Chem. Ann.*, cccvii, p. 130.

<sup>4</sup> Cf. Erlenmeyer: *Ber. d. deutsch. chem. Ges.*, xxxv, p. 2483, 1902.

with sodium amalgam and subsequent removal of the benzoyl group by hydrolysis with hydrochloric acid, the hydrochloride of tyrosine methyl ether was obtained from which the free amino acid was obtained by precipitation with ammonia. The changes may be represented as follows:



That the substance obtained was actually methyl ether was proved by converting it into methyl iodide and tyrosine by boiling with hydriodic acid.

The details of the method employed are as follows: A mixture of anisic aldehyde (1 mol.), powdered hippuric acid (1 mol.), powdered freshly fused sodium acetate (1 mol.) and acetic anhydride (3 mols.) is warmed on the water bath for half an hour. The formation of yellow crystals is soon obvious and at length the whole mixture becomes almost solid. Water is then added and the precipitate filtered off and washed with 80 per cent alcohol. The crude substance is almost pure but is advantageously crystallized from ethyl acetate or acetic acid containing a little water or alcohol. The yield of recrystallized substance is about 80 per cent of the theoretical amount. The crystals are composed of rosettes of golden



yellow needles, m. p.  $158^{\circ}$ , practically insoluble in water, very sparingly soluble in hot alcohol, readily soluble in hot ethyl acetate or acetic acid, but sparingly soluble at the ordinary temperature.

**Analysis:**

0.1364 gm. substance dried at  $100^{\circ}$  gave 0.3666 gm.  $\text{CO}_2$  and 0.0600 gm.  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{17}\text{H}_{15}\text{O}_3\text{N}$ :	Found:
C.....	73.12 per cent	73.29 per cent
H.....	4.66 per cent	4.88 per cent

On dissolving the yellow "azlactone" just described in warm 5 per cent caustic soda and precipitating with hydrochloric acid, colorless  $\alpha$ -benzoylamino-paramethoxycinnamic acid is readily obtained in practically theoretical amount. The acid is best crystallized from aqueous acetone and is obtained in the form of long needles melting at  $230\text{--}232^{\circ}$  with formation of a yellow substance. The melting point is not perfectly sharp, since slow decomposition sets in above  $220^{\circ}$  with formation of a yellow substance which not improbably may be the previously described azlactone from which the acid is obtained. Benzoylamino-paramethoxycinnamic acid is very readily soluble in acetone, moderately soluble in acetic acid, sparingly soluble in alcohol and almost insoluble in water.

**Analysis:**

0.1707 gm. substance dried at  $100^{\circ}$  gave 0.4293 gm.  $\text{CO}_2$  and 0.0827 gm.  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{17}\text{H}_{15}\text{O}_4\text{N}$ :	Found:
C.....	68.69 per cent	68.59 per cent
H.....	5.05 per cent	5.38 per cent

The methyl ether of benzoyltyrosine is obtained by the reduction of the last mentioned substance in the following way:<sup>1</sup> Benzoylamino-paramethoxycinnamic acid (50 gms.) is suspended in ten parts of water contained in a thick-walled flask and fifteen times its weight of 2.5 per cent sodium amalgam is added fairly rapidly. The solution is placed in a warm place ( $25\text{--}30^{\circ}$ ) and allowed to stand over night. One hundred cc. of 33 per cent caustic

<sup>1</sup> The conditions are essentially those adopted by Fischer for the preparation of benzoyltyrosine. *Ber. d. deutsch. chem. Ges.*, 1899, p. 3638.

soda solution is then added to the aqueous solution and the whole boiled for half an hour to destroy any unreduced acid. On cooling and acidifying with hydrochloric acid a gummy mass of crude methyl ether of benzoyltyrosine is precipitated. On standing in a cold place the substance slowly hardens. For the preparation of tyrosine methyl ether it is unnecessary to purify the benzoyl derivative, as it is more economical to purify the product of its hydrolysis. The methyl ether of benzoyltyrosine is best purified by repeated crystallization from 80 per cent acetic acid and is obtained in the form of clear, thin, highly refractive regular hexagonal plates melting at 136–137°. It is very sparingly soluble in water, readily soluble in acetic acid.

**Analysis:**

0.1735 gm. substance dried at 100° gave 0.4335 gm.  $C_2O$  and 0.0939 gm.  $H_2O$ .

	Calculated for $C_{17}H_{17}O_4N$ :	Found:
C.....	68.23 per cent	68.14 per cent
H.....	5.72 per cent	6.01 per cent

The hydrolysis of benzoyltyrosine methyl ether is effected by boiling the acid for six hours with fifty times its weight of 20 per cent hydrochloric acid. The hydrolysis is slow but eventually the whole of the acid is dissolved. The solution is concentrated and then allowed to cool in order to allow the benzoic acid to separate. The filtrate is concentrated to a syrup which crystallizes on cooling owing to the separation of the hydrochloride of tyrosine methyl ether. The syrup is dissolved in a little cold water and filtered from any insoluble matter. Ammonia is then added drop by drop until present in very slight excess. The methyl tyrosine is obtained as an almost colorless precipitate of small clusters of prisms. If any large excess of ammonia be present, it should be removed by allowing the solution to stand in a warm place. Tyrosine methyl ether is very sparingly soluble in water, readily soluble in ammonia or hydrochloric acid, sparingly soluble in acetic acid, even on boiling. It is best purified by dissolving in acid and precipitating with ammonia or sodium acetate and crystallizes in small star-shaped platelets and prisms melting at about 295° (uncorr.) with decomposition. The acid has a pronounced sweet

taste and as would be expected gives no reaction with Millon's reagent. In other respects it resembles tyrosine very closely.

#### Analysis:

0.1563 gm. substance dried at 100° gave 0.3535 gm. CO<sub>2</sub> and 0.0965 gm. H<sub>2</sub>O.

	Calculated for C <sub>10</sub> H <sub>13</sub> O <sub>3</sub> N:	Found:
C.....	61.54 per cent	61.68 per cent
H.....	6.67 per cent	6.86 per cent

On boiling methyl tyrosine (1 gram) with fuming hydriodic acid (5 grams) and a little red phosphorus, methyl iodide is evolved and on evaporating the solution, dissolving the residue in water and neutralizing with ammonia, a practically quantitative yield of tyrosine is obtained.

#### *Fate of Paramethoxyphenylalanine (Methyl Tyrosine) in the Body.*

Three separate experiments were made to determine the fate of paramethoxyphenylalanine. In two of these experiments 4 grams of the substance were converted into the very readily soluble hydrochloride, mixed with chopped meat and fish and given to a cat weighing about 2.5 kilos. In the third experiment 6.0 grams of the free amino-acid were mixed with chopped meat and consumed within twenty-four hours by a cat weighing 3.5 kilos. Since the results in all the three cases were essentially the same, a description of the last experiment will suffice. In each case an examination of the feces failed to reveal the presence of unabsorbed substance.

Part of the urine passed during the forty-eight hours following the consumption of the amino-acid was acidified with acetic acid and allowed to stand in a cool place. No precipitation of methyl tyrosine took place, nor was any obtained on concentrating the urine. When the very sparing solubility of methyl tyrosine is considered, it must be concluded that no considerable amount of unchanged amino-acid was present in the urine. Confirmation of this conclusion was afforded by the results of the titration of the urine (after removal of ammonia) in the presence of formaldehyde, according to the method for the estimation of amino-acids described by W. Frey and A. Gigon.<sup>1</sup> The results obtained from the urines

<sup>1</sup> *Biochemische Zeitschrift*, xxii, p. 309, 1909.

## Decomposition of Tyrosine

passed before and after administration of the methoxyphenyllanine show a slight increase in the amino-acid nitrogen.

	Total N.	Amino-acid N.
Urine before administration of methoxyphenylalanine . . . . .	2.148 per cent	0.026 per cent
Urine passed after administration of methoxyphenylalanine (6.0 grams.) . . . . .	2.380 per cent	0.04 per cent

The remainder of the urine was acidified with phosphoric acid and extracted with ether in a continuous extraction apparatus. The ethereal extract contained a sparingly soluble oily acid, which was eventually purified as follows: The crude extract was dissolved in a slight excess of caustic soda solution and extracted with ether. The alkaline aqueous solution was separated, heated to remove ether and then acidified. An acid was precipitated which, however, did not crystallize. It was filtered off and well washed with water and then dissolved in aqueous ammonia. The solution of the ammonium salt was warmed to remove excess of ammonia and then precipitated with silver nitrate. An almost white, stable silver salt was obtained which was successively washed with water, alcohol and ether. On analysis the silver salt gave numbers agreeing with those required for silver methoxyphenyl acetate:

## Analysis:

Prep. I: 0.2232 gm. gave 0.0882 gm. Ag. = 39.52 per cent.

Prep. II<sup>1</sup>: 0.1057 gm. gave 0.0421 gm. Ag. = 39.83 per cent.

A third portion of the silver salt was decomposed with dilute nitric acid. An oily acid was at first obtained, which slowly solidified and yielded crystals, m.p. 85–86°, identical with paramethoxyphenylacetic acid prepared for comparison.

Paramethoxyphenylacetic acid was prepared for purposes of comparison by dissolving parahydroxyphenylacetic acid in 10 per cent caustic soda solution and adding an excess of dimethyl sulphate. The mixture was then heated to boiling and more caustic

<sup>1</sup> A separate preparation from a different experiment from that which furnished Prep. I.

soda solution added by degrees until complete solution was effected. On cooling, filtering and acidifying with hydrochloric acid a practically theoretical yield of paramethoxyphenylacetic acid was obtained. The substance crystallized in platelets melting at 85–86° and was identical with the product obtained by Canizzaro from paramethoxybenzylcyanide.<sup>1</sup> Paramethoxyphenylacetic acid appears not to have been previously obtained by the methylation of hydroxyphenylacetic acid.

The ethereal extracts of the urines from which the methoxyphenylacetic acid was separated were specially examined for any hydroxy-aromatic acid, or uramido derivative of methyltyrosine and for any glycocoll derivative of methoxyphenylacetic acid, but no indication of the presence of any of these substances could be obtained.

*Fate of Parahydroxybenzaldehyde in the Body.* Two grams of parahydroxybenzaldehyde were mixed with fish and given to a cat weighing 3.5 kilos. On the following day the same doses were again administered. Half of the urine passed during a forty-eight hour period of collection was examined for hydroquinone or its derivatives by boiling with dilute hydrochloric acid for half an hour, cooling, extracting with ether and testing the ether residue for hydroquinone with alkaline silver and copper solutions. The result was negative. The remainder of the urine was concentrated acidified with phosphoric acid and thoroughly extracted with ether. On evaporation of the ether a crystalline residue weighing 1.3 grams was obtained (equivalent to 2.6 grams for the whole quantity of urine). The residue consisted of a mixture of about equal quantities of parahydroxyhippuric acid and parahydroxybenzoic acid. The substances were separated by treatment with dry, alcohol-free ether, in which the hydroxybenzoic acid is very much more soluble than the hydroxyhippuric acid. Both substances were finally purified by repeated crystallization from water.

The parahydroxyhippuric acid crystallizes from water in well-formed crystals, m. p. 228°, agreeing with the properties of the substance described by Schotten.<sup>2</sup>

<sup>1</sup> Liebig's *Annalen*, cxvii, p. 243.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, vii, p. 26.

## Decomposition of Tyrosine

## Analysis:

0.1777 gram gave 0.0783 gm.  $\text{H}_2\text{O}$  and 0.3625 gm.  $\text{CO}_2$ .

	Calculated for $\text{C}_9\text{H}_9\text{O}_4\text{N}$ :	Found:
C.....	55.38 per cent.	55.64 per cent
H.....	4.62 per cent	4.89 per cent

The parahydroxybenzoic acid after recrystallization melted at  $213^\circ$ .

## Analysis:

0.2148 gm. substance dried at  $100^\circ$  gave 0.0860 gm.  $\text{H}_2\text{O}$  and 0.4775 gm.  $\text{CO}_2$ .

	Calculated for $\text{C}_7\text{H}_6\text{O}_2$ :	Found:
C.....	60.87 per cent.	60.62 per cent.
H.....	4.34 per cent.	4.45 per cent.

*Fate of the Sodium Salt of Salicylic Aldehyde.* Aqueous solutions of the sodium salt of salicylic aldehyde were prepared by dissolving weighed amounts of the aldehyde in the calculated amount of normal caustic soda. The solution of sodium salt equivalent to 1.5 gm. of the aldehyde was given with 100 cc. of water by mouth to a rabbit. The urine passed during the following twenty-four hours contains very little if any unchanged aldehyde, but on acidifying and extracting with ether 0.6 gram of crystals were obtained, which were somewhat deeply pigmented. On recrystallization from boiling benzene, pure salicylic acid, m.p.  $157^\circ$ , was readily obtained. The crude crystals contained only traces of nitrogen, so that no large amount of salicyluric acid was present. No indications of the presence of catechol or its derivatives could be obtained either before or after boiling the urine with acid. In a second experiment 3.0 grams of the aldehyde was given in aqueous solution in the form of the sodium salt to a rabbit. The animal died after five hours, but the urine contained much salicylic acid—0.7 gram was obtained in the pure crystalline form. In a third experiment the sodium salt of salicylic aldehyde (2.0 grams) was given subcutaneously to a cat. The results were similar to those of the preceding experiments. No catechol nor derivatives of it could be detected.

**THE FATE OF INACTIVE TYROSINE IN THE ANIMAL BODY TOGETHER WITH SOME OBSERVATIONS UPON THE DETECTION OF TYROSINE AND ITS DERIVATIVES IN THE URINE. THE SYNTHESIS AND PROBABLE MODE OF FORMATION OF BLEND-ERMANN'S PARA-HYDROXYBENZYLHYDANTOIN.**

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(Received for publication, April 13, 1910.)

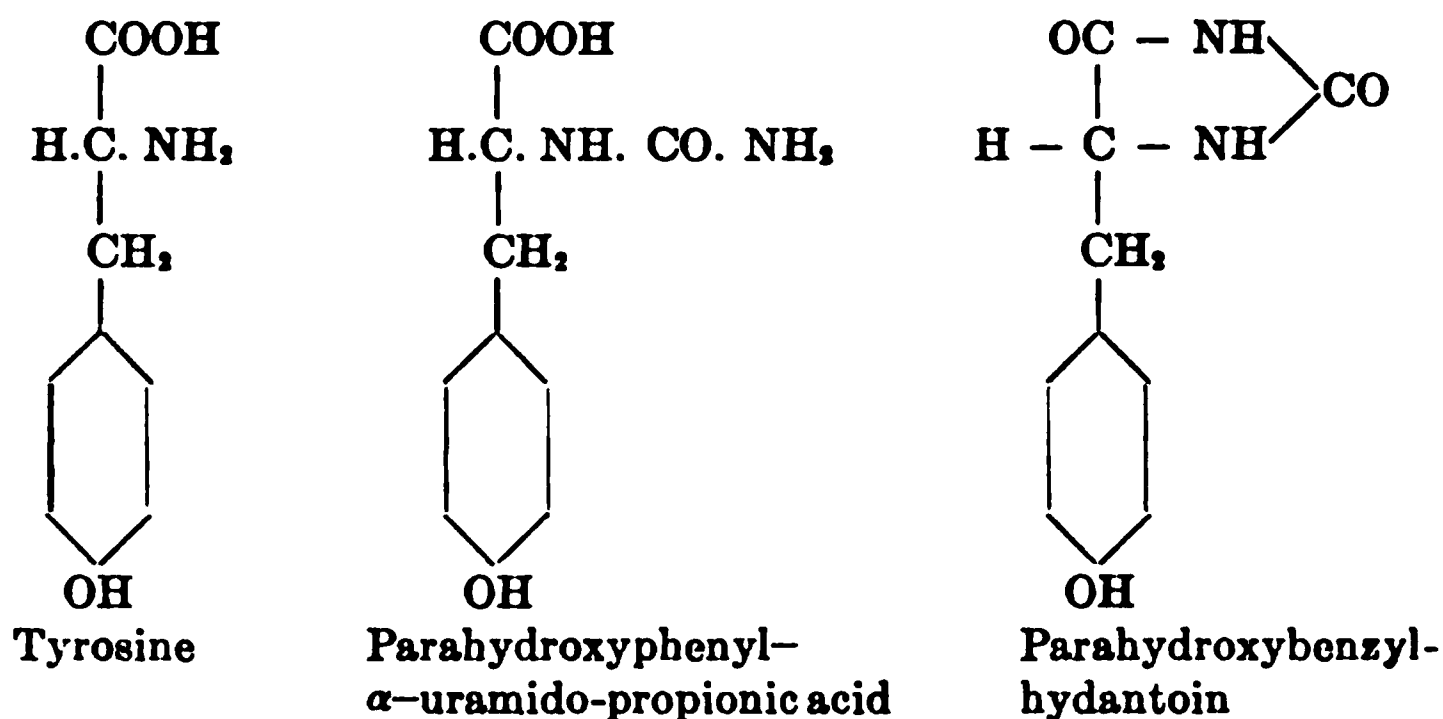
The object of the following paper is to describe certain experiments upon the fate of inactive tyrosine in the body. Wohlge-muth<sup>1</sup> in a preliminary communication records a single experiment in which 8.0 grams of inactive tyrosine was given to a rabbit. From the urine passed subsequently 1.7 gram of tyrosine was recovered of which about 75 per cent was the dextro-form. I have been able to confirm this result by a number of additional experiments in all of which it was possible to demonstrate a selective decomposition of the two stereoisomeric forms constituting the inactive acid. The naturally occurring lævo-acid was in every case more readily decomposed but the rates of decomposition of the *d* and *l* forms cannot be very widely different since, when smaller doses of the inactive acid are given, no unchanged tyrosine may be found in the urine.

One of the main objects of my experiments was to try to detect in the urine of animals which had received large doses of tyrosine, some intermediary product of the catabolism of the amino-acid in question. Special attention was given to the possible formation of homogentisic acid, but in no case could any be detected. On concentrating the urines obtained from some of these experiments, and subsequently acidifying strongly with phosphoric acid and

<sup>1</sup>*Ber. d. deutsch. chem. Ges.*, xxxviii, p. 2064, 1905.

extracting with ethyl acetate in a continuous extractor, it was found that tyrosine derivatives were present in the ethyl acetate extract. The extracts were accordingly submitted to steam distillation, decolorized with charcoal, filtered and slowly concentrated. By this means a small amount of a sparingly soluble crystalline substance was obtained which proved to be dextro-rotatory para-hydroxybenzylhydantoin<sup>1</sup> together with a little of the inactive substance.

The mother liquor from which these crystals were obtained could not be induced to crystallize even on prolonged standing. It was therefore boiled with a little hydrochloric acid, when a further crop of crystals was readily obtained identical with those previously separated. The second crop of crystals was doubtless formed by the action of the acid upon the very soluble and difficultly crystallizable para-hydroxyphenyl- $\alpha$ -uramidopropionic acid. The relation of these substances to tyrosine is evident from the following formulæ:



Parahydroxybenzylhydantoin<sup>2</sup> has been described by Blendermann<sup>3</sup> and by Lippich.<sup>4</sup> The former obtained it from the urine of rabbits which had received large doses of tyrosine and recorded the melting-point as 275°–280°. Lippich obtained the substance by

<sup>1</sup> This substance is generally referred to under the name of tyrosinhydantoin. It is clear, however, that this name is inapplicable and misleading.

<sup>2</sup> Presumably the laevo-compound.

<sup>3</sup> *Zeitschr. f. physiol. Chem.*, vi, p. 234.

<sup>4</sup> *Ber. d. deutsch. chem. Ges.*, xli, p. 2973.



heating parahydroxyphenyl- $\alpha$ -uramidopropionic acid with mineral acids and gives the melting-point as 242°–245°, without, however, commenting upon the discrepancy in the two observations. My own results comprise many observations on several different preparations of the racemic and lævo and dextro compounds, and although the melting-points vary slightly with the speed of heating and slight decomposition sets in two or three degrees before actual melting occurs, all preparations of each of the three substances melted within the limits of 258°–263°. In the experimental part of this paper details are given of a convenient method of synthesis for both the active and inactive substances. The method is based upon the action of potassium cyanate solution upon tyrosine and the direct conversion without isolation of the uramido-acid thus formed, into the desired hydantoin by the action of boiling hydrochloric acid.

In spite of the fact that *p*-oxyphenyl- $\alpha$ -uramidopropionic acid and the corresponding hydantoin had been obtained from the urines of cats which had received large doses of tyrosine, there was a possibility that these substances might be formed during the analysis from unchanged tyrosine present in the urine. The possibility of such a change is obvious in the light of Lippich's experiments in which tyrosine on boiling with excess of urea solution was practically completely converted into the corresponding uramido-acid. Since in the preceding experiments the urine containing unchanged tyrosine had been concentrated without acidifying, the conditions for the formation of the uramido-acid were undoubtedly present. Accordingly new experiments were instituted in which the urine as soon as passed was acidified with acetic acid, and when it was necessary to concentrate the urine its reaction was constantly maintained distinctly acid. Under these conditions the yield of uramido-acid or of *p*-hydroxybenzylhydantoin was so slight that it was not even possible to identify them satisfactorily. The inference is clear that in the cases in which special precautions to avoid formation during analysis of the uramido-acid were not taken, the greater part of the uramido-acid and of the *p*-hydroxybenzylhydantoin actually isolated was undoubtedly formed after the urine was passed. On reading the account given by Blendermann of the experiments which led him to the discovery of parahydroxybenzylhydantoin, it is certain that the conditions for the

formation of this substance outside of the body were present. The demonstration of the formation of parahydroxyphenyl- $\alpha$ -uramidopropionic acid and of parahydroxybenzylhydantoin as products of the metabolism of tyrosine must be considered still lacking.<sup>1</sup>

#### EXPERIMENTAL.

*Fate of Inactive Tyrosine in the Body.* Four separate experiments were made in which quantities of inactive tyrosine varying from 2–10 grams were mixed with chopped meat and given to cats weighing from 2.5 to 3.9 kilos. In the two first experiments the amino-acid was converted into the more soluble hydrochloride before being added to the food; in the other two cases the free amino-acid was administered. After the food containing the tyrosine had been consumed, the cat was removed to a fresh cage, so that there was no possibility of any contamination of the urine with tyrosine-containing food. In no case was any indication obtained of the presence of homogentisic acid in the urine. The quantity of unchanged tyrosine recovered from the urine by crystallization varied from a trace in the first experiment in which 2.0 grams of tyrosine was administered to 0.4 gram in an experiment in which 4.0 grams of tyrosine hydrochloride was consumed. The recovered tyrosine was purified by dissolving it in a small amount of dilute hydrochloric acid, filtering, concentrating, adding excess of ammonia and then allowing the amino-acid to slowly crystallize out as the ammonia evaporated. The tyrosine crystallized in needles and was a mixture of the dextro-rotatory and inactive varieties in the proportion of about 2: 1.

*Rotation:* 0.120 gram substance dissolved in 8 c.c. of 4 per cent hydrochloric acid showed a rotation of  $+0.130^\circ$  in a 1 dm. tube. Hence  $[\alpha]_D^{20} = +8.6^\circ$

Lævo-tyrosine, according to Fischer, has a rotation of 13.2 when dissolved in 4 per cent hydrochloric acid and doubtless the dextro variety would possess the same value.

<sup>1</sup> In the case of the related amino-acid, phenylalanine, the author was able to demonstrate the formation in the animal body of the corresponding uramido acid. At least the substance crystallizes directly from the urine without any concentration.

In two experiments the urine, after filtering off unchanged tyrosine, was concentrated on the water-bath without previously acidifying. The concentrated urine was then acidified with phosphoric acid and extracted with ethyl acetate in a continuous extractor. The ethyl acetate extracts after distillation in steam and treatment with charcoal gave on concentration a small yield of a sparingly soluble crystalline substance which after recrystallization was obtained in the form of colorless needles melting at 258°–260°. The total quantity of substance obtained from 8.0 grams of tyrosine was only 0.09 gram. The substance proved to be dextro-*para*hydroxybenzylhydantoin and was identified in every respect save that of sign of rotation with the corresponding substance prepared from *laevo*-tyrosine (see later).

*Rotation:* 0.045 gram dissolved in 6.5 c.c.  $\frac{N}{1}$  NaOH solution had a rotation of 1.01°.  $[\alpha]_D^{20} = + 146^\circ$ .

*Analysis:*

0.0701 gram gave  $\text{NH}_3 = 0.0097\text{g}$ . N = 13.8 per cent N  
 $\text{C}_{10}\text{H}_{10}\text{O}_3 \text{ N}_2$  requires 13.6 per cent N

The small amount of available material precluded the possibility of a more complete analysis of the substance. When a minute quantity of the dextro substance was mixed with an equal weight of the *laevo*-hydantoin prepared from natural tyrosine and the mixture crystallized from water, the crystals melted at the same temperature as inactive *para*-hydroxybenzylhydantoin prepared from inactive tyrosine (see later).

The mother liquor separated from the crystals obtained from the ethyl acetate extract could not be induced to crystallize even on prolonged standing. As it appeared not unlikely that *p*-hydroxyphenyl- $\alpha$ -uramidopropionic acid might be present and as this substance is difficult to crystallize unless quite pure, it was decided to boil the syrupy residue with a little dilute sulphuric acid in order to convert the uramido-acid into the sparingly soluble hydantoin. On cooling the solution crystals separated which were contaminated with oily impurities. The crystals were readily purified by washing them with a little ether and then recrystallizing from boiling water. The crystals melted at 258°–260° and had all the properties of *p*-hydroxybenzylhydantoin. Only 0.07 gram of pure crys-

tals were recovered from the two experiments. The hydantoin was a mixture of the racemic and dextro-forms in which the former predominated.

Owing to the possibility that the uramido-acid and hydantoin separated as just described, might have formed during analysis from unchanged tyrosine present in the urine, two additional experiments were made in which the urine was acidified with acetic acid as soon as voided. In these experiments 4.0 and 10.0 grams of inactive tyrosine were consumed but on analysis of the urine after separation of small amounts of dextro-rotatory tyrosine, no definite indications could be obtained of the presence of either *p*-hydroxyphenyl- $\alpha$ -uramidopropionic acid or of *p*-hydroxybenzyl-hydantoin.

*Synthesis of Inactive Para-hydroxybenzylhydantoin.* Inactive tyrosine (5.0 grams) is suspended in actively boiling water (25 cc.) and potassium cyanate is rapidly added in small portions until the whole of the tyrosine is dissolved. Hydrochloric acid (50 cc. of 10 per cent HCl) is then added and the whole boiled under a reflux condenser for half an hour. A little charcoal is then added and on filtering and cooling a good yield (4.5 grams) of the desired compound is obtained. The substance is apt to crystallize in hard nodules made up of prisms. It is moderately soluble in hot water, sparingly in cold and sparingly soluble in alcohol and ether but readily dissolves in caustic soda solution. Its aqueous solutions react with Millon's reagent slowly in the cold but readily on warming. The melting-point varies somewhat with the speed of heating and also with the amount of substance taken. With a moderate rate of heating and using a small amount of substance in a narrow capillary tube, the melting point of many different preparations lay always between 258°–260° (uncorr.), the substance showing some signs of decomposition two or three degrees before melting.

*Analysis:*

0.1822 gram gave 0.0818 gm. H<sub>2</sub>O and 0.3889 gm. CO<sub>2</sub>  
 0.1763 " " NH<sub>3</sub> = 0.02394 gm. N

	Calculated for C <sub>10</sub> H <sub>10</sub> O <sub>3</sub> N <sub>2</sub> :	Found:
C.....	58.25 per cent	58.21 per cent
H.....	4.85 per cent	4.99 per cent
N.....	13.59 per cent	13.58 per cent

*Lævo-Para-hydroxybenzylhydantoin.* This substance was prepared in the same way as in the case of the inactive substance, using a specimen of recrystallized lævo-tyrosine in place of the inactive amino-acid. The l-tyrosine was prepared from products of autolysis of the cell juice from ox kidneys.<sup>1</sup> Its optical rotation was observed in 4 per cent hydrochloric acid and the result agreed well with that recorded by E. Fischer.

$$C = 1.650; l = 1.0; \alpha = -0.21$$

$$\text{Hence } [\alpha]_D^{20} = -12.7^\circ$$

The hydantoin prepared from this tyrosine crystallized from water in aggregates of white needles melting at  $259^\circ$ – $262^\circ$  (uncorr.) closely resembles the inactive compound as regards solubility and reaction with Millon's reagent.

*Analysis:*

0.1666 gram. gave  $\text{NH}_3 = 0.02275$  gm. N = 13.66 per cent

Calculated for  $\text{C}_{10}\text{H}_{10}\text{O}_3$ : N = 13.58 per cent

The hydantoin is readily soluble in caustic soda solution and the solution shows a high optical activity.

Prep. I: C = 3.28 per cent in  $\frac{N}{1}$  NaOH;  $l = 1$  dm.;  $\alpha = -4.65^\circ$

Prep. II: C = 1.866 per cent in  $\frac{N}{1}$  NaOH;  $l = 2$  dm.;  $\alpha = -5.32^\circ$

$$[\alpha]_D^{20} = -142^\circ$$

If the alkaline solution of the hydantoin is observed from time to time in the polarimeter it is noticed that the rotation steadily diminishes until the solution finally becomes optically inactive. In the experiment recorded above (Prep. II) the angle of rotation had reduced to about half the initial value in four hours and after twenty-four hours had fallen to zero. On acidifying the alkaline optically inactive solution a precipitate of inactive *p*-hydroxybenzylhydantoin (m.p.  $258^\circ$ – $260^\circ$ ) is at once obtained. This remarkable type of racemization has been further investigated and appears to be characteristic of the hydantoins. It is probably to be explained on the basis of tautomeric change.<sup>2</sup> Para-hydroxyphenyl-

<sup>1</sup> *Journ. of Physiol.*, xxx, p. 84.

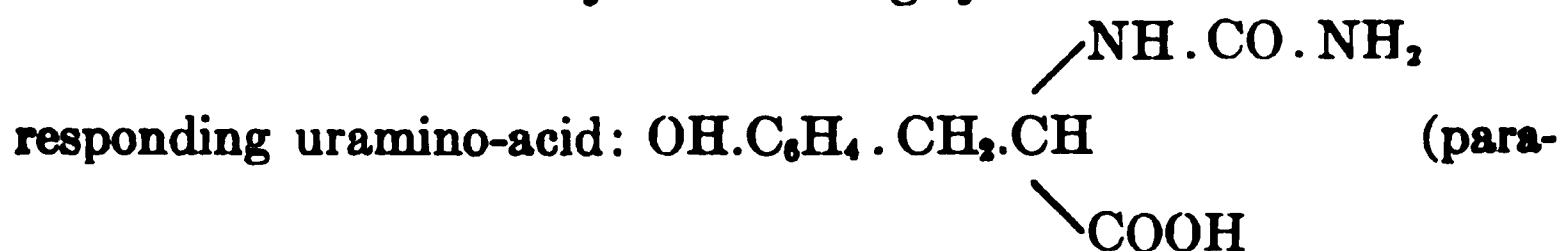
<sup>2</sup> The results of a more extended study of the change will be published elsewhere.

$\alpha$ -uramidopropionic acid does not undergo any similar racemisation under like conditions.

#### SUMMARY.

Inactive tryosine administered to cats undergoes selective decomposition in such a manner that any tyrosine excreted in the urine contains more of the dextro than of the naturally occurring lævo variety. This result confirms Wohlgemuth's experiments.

When neutral or alkaline urine containing tyrosine is evaporated on the water-bath the tyrosine is largely converted into the cor-



hydroxyphenyl- $\alpha$ -uramidopropionic acid). This substance on subsequent treatment with acids readily loses water giving the hydantoin derivative:  $\text{OH} \cdot \text{C}_6\text{H}_4 \cdot \text{CH}_2 \cdot \text{CH} \begin{array}{l} \nearrow \text{NH} - \text{CO} \\ \searrow \text{CO} - \text{NH} \end{array}$  (para-hydroxy-

benzylhydantoin) described by Blendermann and obtained by him from the urines of rabbits which had received very large doses of tyrosine. It is probable that Blendermann's substance was largely produced as the result of the analytical procedures to which the urine was subjected. There is no evidence that the hydantoin is an intermediary product of the catabolism of tyrosine.

In order to detect tyrosine in urine it is therefore important to avoid heating the urine unless acidified. In some experiments upon the urine from animals which had received moderately large doses of inactive tyrosine and in which the urine was not acidified before concentration, it was found possible to isolate small quantities of dextro-rotatory para-hydroxyphenyl- $\alpha$ -uramidopropionic acid and the corresponding hydantoin in addition to dextro-rotatory tyrosine. When precautions were taken to avoid conditions during analysis which might give rise to the  $\alpha$ -uramido acid and hydantoin practically none could be detected.

When tyrosine is administered to cats in such large amounts that much appears unchanged in the urine no evidence could be

obtained of the simultaneous excretion of homogentisic acid or other di-hydroxy-aromatic acid.

A convenient method for the synthesis of lævo and racemic para-hydroxybenzylhydantoin is described, together with some observations upon the catalytic racemisation of the first of these substances—the *L*-compound—into the inactive compound by means of alkali at room temperature.





# THE MODE OF OXIDATION OF PHENYL DERIVATIVES OF FATTY ACIDS. A CORRECTION.

By H. D. DAKIN.

(From the Laboratory of Dr. C. A. Herter, New York.)

(Received for publication, April 13, 1910.)

In a recent paper<sup>1</sup> the results of the investigation of the fate in the animal organism of a number of derivatives of phenylpropionic acid were recorded. Among the substances examined was the product described by Posen<sup>2</sup> obtained by the action of ammonia upon phenyl- $\beta$ -bromopropionic acid, which was believed to be phenyl- $\beta$ -alanine in accordance with the statements in the literature. Recently, however, Posner<sup>3</sup> has found that the substance obtained by him through the action of hydroxylamine upon cinnamic ester was not phenyl- $\alpha$ -alanine, as he at first supposed, but phenyl- $\beta$ -alanine and that Posen's compound had a different constitution. The latter compound was shown to be the amide of phenyl- $\beta$ -oxypropionic acid and this result I have been able to confirm. The recognition of the erroneous constitution previously assigned to Posen's compound made it necessary to correct the previously recorded statements as to the fate of phenyl- $\beta$ -alanine since they actually apply to the amide of phenyl- $\beta$ -oxypropionic acid. With this end in view experiments have been made upon the fate in the animal organism of pure phenyl- $\beta$ -alanine prepared by Posner's<sup>4</sup> method.

The results may be summarized as follows: The urines of cats or small dogs which have received phenyl- $\beta$ -alanine in doses of about 3.0 grams, either subcutaneously in the form of the very soluble hydrochloride or by mouth as the free amino-acid, contain

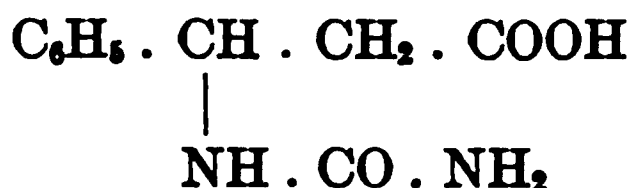
<sup>1</sup> This *Journal*, vi, p. 235, 1909.

<sup>2</sup> Liebig's *Annalen*, cxcv, p. 144.

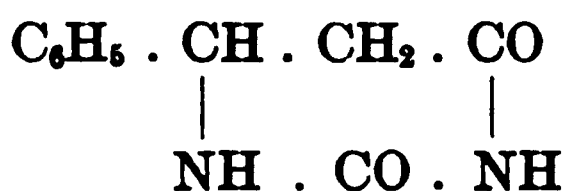
<sup>3</sup> *Ber. d. deutsch. chem. Gesellschaft*, xxxviii, p. 2316.

<sup>4</sup> *Ibid.*, p. xxxvi, 4305.

much unchanged amino-acid. If the neutral urine be concentrated upon the water bath much of it may be converted by interaction with urea into phenyl- $\beta$ -uramido-propionic acid:



and this by the subsequent action of acids may be converted in the course of the analysis into the corresponding anhydride, phenyldihydrouracil,



If the urine is acidified as soon as passed and conditions for the conversion outside the body of phenyl- $\beta$ -alanine into the uramido acid be avoided, practically none of the latter substance is found. No indications could be obtained of the formation of phenyl- $\beta$ -oxypropionic acid, acetophenone or hippuric acid.

The behavior of phenyl- $\beta$ -alanine in the body is therefore in marked contrast to that of the corresponding hydroxy and ketonic acids,  $\text{C}_6\text{H}_5 \cdot \text{CH}(\text{OH}) \cdot \text{CH}_2 \cdot \text{COOH}$  and  $\text{C}_6\text{H}_5 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{COOH}$ , both of which are converted into hippuric acid.

In addition to the foregoing correction, I wish to take this opportunity to amplify a statement in my paper upon the fate of phenylvaleric acid in the animal body<sup>1</sup>. It was stated that in Knoop's paper upon the same subject "no attempt is made to picture the mechanism of the reaction" involved in the oxidation of this acid, although "it is clearly to be inferred that Knoop recognized the possibility of the oxidation being indirect." The fact is, however, that Knoop actually refers to the possibility of the formation of phenylpropionic acid as an intermediate step in the catabolism of phenylvaleric acid,<sup>2</sup> and I much regret that by an oversight this was not specifically mentioned in my original paper.

<sup>1</sup> *This Journal*, vi, p. 221.

<sup>2</sup> *Der Abbau aromatischer Fettsäuren im Thierkörper*, p. 41. Freiburg: Ernst Kuttruff, 1904.

## EXPERIMENTAL.

An attempt was made to estimate approximately the amount of unchanged phenyl- $\beta$ -alanine in the urine of cats which had received 3.0 grams of the amino-acid mixed with chopped meat. The method was based upon titration of the urine, after removal of ammonia, in the presence of excess of formaldehyde, and the details were those adopted by Frey and Gigon.<sup>1</sup> In two experiments the results indicated 2.1 and 2.4 grams of amino-acid calculated as phenyl- $\beta$ -alanine, while the normal amount of amino-acid during the same period was only about 5 per cent of these amounts. It is probable, therefore, that about 75 per cent of the phenyl- $\beta$ -alanine consumed was excreted unchanged.

When the urine from animals that had received phenyl- $\beta$ -alanine was concentrated without acidification and then subsequently made acid with phosphoric acid and extracted with ethyl acetate, the ethyl acetate extract was found to contain phenyl- $\beta$ -uramidopropionic acid. The ethyl acetate extract was purified in the same method as that used in the case of tyrosine referred to in the previous paper. On concentrating the steam-distilled aqueous solution crystals were readily obtained which after recrystallization from water melted at 194–195°. The product proved to be identical in all respects with phenyl- $\beta$ -uramidopropionic acid prepared synthetically. The yield of crystalline substance under favorable conditions amounted to about 10 per cent of the phenyl- $\beta$ -alanine consumed, but was frequently less.

*Analysis:*

0.1498 gram gave  $\text{NH}_3$  = 0.0203 gm. N = 13.55 per cent N.

$\text{C}_{10}\text{H}_{13}\text{O}_2\text{N}_2$  requires 13.46 per cent N.

In one experiment in which 3.0 grams of phenyl- $\beta$ -alanine hydrochloride had been given subcutaneously to a cat weighing 3.2 kilos, the urine on analysis furnished a small quantity of very sparingly soluble wart-like clumps of crystals which separated from the aqueous solution before the latter was sufficiently concentrated for the uramido-acid to crystallize out. On recrystallization from water the substance was readily purified and melted at 216–217° and

<sup>1</sup> *Biochemische Zeitschrift*, xxii, p. 309.

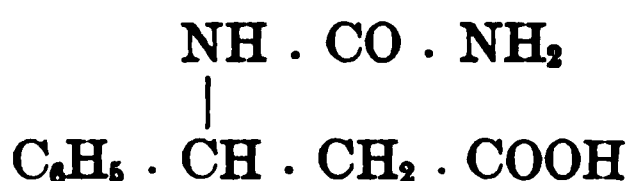
proved to be the anhydride of the previously described uramido-acid, phenyldihydrouracil. It was identical in all respects with the synthetically prepared substance.

*Analysis:*

0.1008 gram gave  $\text{NH}_3 = 0.0148 \text{ gm. N} = 14.67 \text{ per cent.}$

$\text{C}_{10}\text{H}_{10}\text{O}_2\text{N}_2$  requires 14.74 per cent.

*Preparation of Phenyl- $\beta$ -Uramidopropionic Acid*



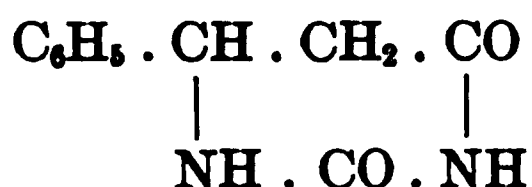
Phenyl- $\beta$ -alanine (3.0 grams), potassium cyanate (3.0 grams) and water (15 c.c.) are stirred together and the mixture evaporated to dryness on the water-bath. The residue is taken up in hot water, filtered and the crude uramido-acid precipitated by the addition of hydrochloric acid. The substance is purified by recrystallization from hot water in which it is readily soluble and separates on cooling in the form of needles or small hexagonal prisms. The yield is about 70 per cent of the theoretical amount. The melting point varies with the rate of heating, but when moderately rapidly heated the substance melts constantly at  $194\text{--}195^\circ$  with decomposition. Posner has recently described the same substance and gives  $191^\circ$  as the melting point.<sup>1</sup>

*Analysis:*

0.1144 gram gave 0.1790 gram  $\text{CO}_2$  and 0.0605 gram  $\text{H}_2\text{O}$ .

Calculated for $\text{C}_{10}\text{H}_{12}\text{O}_3\text{N}_2$ :		Found:
C.....	57.69 per cent.	57.38 per cent.
H.....	5.77 per cent.	5.88 per cent.

*Preparation of Phenyldihydrouracil.*



Posner<sup>2</sup> obtained this substance by heating phenyl- $\beta$ -uramidopropionic acid above its melting point, but it is more easily

<sup>1</sup> *Ber. d. deutsch. chem. Ges.*, xxxviii, p. 2323.

<sup>2</sup> *Loc. cit.*

obtained in excellent yield by simply boiling the latter substance for a short time with ten parts of 10 per cent hydrochloric acid. The substance crystallizes from water in fine needles or platelets, melting sharply at 216-217°. It is sparingly soluble in cold water. Cold caustic soda solution readily dissolves the substance which may be precipitated unchanged on acidifying the solution.

**Analysis:**

0.1981 gram gave 0.4575 g. CO<sub>2</sub> and 0.0962 g. H<sub>2</sub>O.

Calculated for C <sub>10</sub> H <sub>10</sub> O <sub>2</sub> N <sub>2</sub> :		Found:
C.....	63.16 per cent.	62.98 per cent.
H.....	5.26 per cent.	5.39 per cent.



# ON ALKYLAMINES AS PRODUCTS OF THE KJELDAHL DIGESTION.

By C. C. ERDMANN.

(From the Chemical Laboratory of the McLean Hospital for the Insane,  
Waverley, Mass.)

(Received for publication, April 25, 1910.)

In a recent paper Folin<sup>1</sup> describes a method for the detection of alkylamines in urine. The method he used for their detection is based on the formation of isonitril by adding alcoholic potash and chloroform to the distillate of urine, which after digestion according to Kjeldahl has been concentrated to small volume. The test, however, is only a qualitative one, and so sensitive that with the smallest amount of primary amines the characteristic odor of isonitril will be obtained.

For the continuation of this work it seemed necessary to find a method for the quantitative estimation of alkylamine in presence of ammonia.

Maurice François's<sup>2</sup> observation that the purification of methylamine from ammonia can be effected by means of yellow oxide of mercury suggested the idea to use mercuric oxide for the separation of ammonia and alkylamine.

When applied to mixtures of organic base and ammonia in presence of caustic alkali and alkali carbonate the oxide of mercury will absorb the ammonia, while the organic base remains in solution. Thus it is possible to determine either of the constituents and to calculate the other. Because of greater accuracy as well as simplicity the determination of the organic base in the liquid was preferred. The method is carried out as follows:

<sup>1</sup> On the Occurrence and Formation of Alkylurea and Alkylamines, *This Journal*, iii, p. 83, 1907.

<sup>2</sup> Maurice François: *Compt. rend. Acad. Sci.*, cxliv, p. 567.

An amount of substance equal to about 20-30 cc. of  $\frac{N}{10}$  nitrogen is digested with sulphuric acid and catalyser and distilled into a graduated flask of 250 or 500 cc. capacity containing excess of  $\frac{N}{10}$  acid. The distillate is titrated to ascertain the amount of total nitrogen. To the neutral solution containing organic base and ammonia 5-10 cc. of an alkaline mixture consisting of 20 per cent sodium hydrate and 30 per cent sodium carbonate, is added. The flask is filled to the mark with distilled water and for the absorption of the ammonia 0.1 gram of yellow oxide of mercury for each cc. of  $\frac{N}{10}$  ammonia is required. After shaking for one hour, light being excluded, the flasks are set aside for 12 hours to allow the mercuric oxide to settle.

The separation of the liquid from the mercuric oxide is effected by forcing the clear,<sup>1</sup> colorless liquid through an absorbent cotton filter by means of a moderate air blast. In 200 or 250 cc. of the filtrate the amount of organic base is determined by distillation and titration. In a solution of ammonium sulphate containing 20 cc. of  $\frac{N}{10}$  ammonia, the latter was absorbed by 2 grams of mercuric oxide (10 cc. alkaline mixture).

Total Nitrogen 20.0 cc.	
Distillation after being shaken with mercuric oxide:	
10.0	10.0
10.0	9.95
<hr/>	
00.0	0.05

A mixture of methylamine and ammonia was then subjected to the same treatment and it was found that the same amount of organic base was obtained in both cases:

Total Nitrogen 18.9    18.9		
Distillation of 200 cc. of the filtrate after separation:		
Organic base in.....	200	8.9            8.85
“            “            “ .....	250	11.10        11.05

In the following determinations methylamine and ammonia were both experimentally determined:

<sup>1</sup> Alizarine used as indicator had been absorbed by the mercuric oxide.



50 cc. of solution:

Total nitrogen.....	20.5
Organic base.....	18.75

---

Ammonia by difference..... 1.75

Ammonia determined in the mercuric oxide residue = 1.6 cc.  $\frac{N}{10}$ .

In 50 cc. of the same solution:

Total nitrogen.....	102.5
Organic base.....	94.

---

Ammonia by difference..... 8.5 cc.  
Experimentally found..... 8.3 cc.

The oxide of mercury was filtered on a Gooch crucible, washed with a dilute solution of the alkaline mixture and transferred to the distilling flask. After being dissolved it was precipitated by potassium sulphide, then alkali added and distilled.

This procedure proved to be rather complicated and did not give satisfactory results. Owing to the amorphous form of mercuric oxide it was difficult to rid it of organic base, while prolonged washing seemed to cause a loss of ammonia. Therefore in further separations the organic base was experimentally determined and the ammonia calculated.

#### METHYLAMINE.

Special attention was paid to the investigations of the behavior of methylamine when digested with sulphuric acid and catalyser.

As some of the substances to be analyzed in the course of this work were such that they would yield methylamine by decomposition, it was necessary to know if the whole amount of organic base could be determined after digestion with sulphuric acid and catalyser.

Therefore the estimation of methylamine was carried out in solutions of the hydrochloride which had been digested for periods ranging from 30 minutes to several hours.

Kahlbaum's methylamine which had served for previous investigations had been found to contain a small amount of ammonia.

10 cc. of a solution containing 21.0 cc. of  $\frac{N}{10}$  nitrogen were separated in a 250 cc. graduated flask by mercuric oxide and alkaline mixture:

Total nitrogen.....	21.0	21.0
Organic base.....	19.4	19.3
	<hr/>	<hr/>
Ammonia.....	1.6	1.7

The presence of ammonia in a methylamine solution can easily be shown by a quantitative test:

Nessler's Reagent forms with methylamine a bright yellow (lemon color) precipitate; if traces of ammonia are present the color of the precipitate changes to yellowish brown.

The substance was purified and the purity proven by concordant total nitrogen and alkylamine determinations:

Total nitrogen.....	18.5	18.5
Organic base.....	18.5	18.5
	<hr/>	<hr/>
Ammonia.....	0	0

This pure preparation was used for the following digestions which were carried out in a lead bath to obtain even temperature.

A thermometer was immersed in the lead and the highest temperature recorded. The duration of digestion could not accurately be stated, as aqueous solutions of methylamine hydrochloride were used requiring some time for concentration.

Solution No. 1 = 15.1 cc.  $\frac{N}{10}$  nitrogen; 20 cc.  $H_2SO_4$ ; temperature  $360^\circ$ ; 30 minutes.

	Catalyser	No Catalyser	
Total nitrogen.....	14.8	14.85	14.9
Organic base.....	14.6	14.75	14.8
	<hr/>	<hr/>	<hr/>
Ammonia.....	0.2	0.1	0.1

Same solution; 20 cc.  $H_2SO_4$ ; temperature  $360^\circ$ ; time, 2 hours.

	Catalyser	No Catalyser	
Total nitrogen.....	15.1	14.9	15.0
Organic.....	14.1	14.1	14.4
	<hr/>	<hr/>	<hr/>
Ammonia.....	1.0	0.8	0.6

Solution No. 2 = 20.65 cc.  $\frac{N}{10}$  nitrogen; 20 cc.  $H_2SO_4$ ; temperature, 370–400°; time, 2 hours.

	Catalyser	No Catalyser
Total nitrogen.....	20.6	20.6
Organic base.....	19.4	19.2
Ammonia.....	1.2	1.4

Same solution; 10 cc.  $H_2SO_4$ ; temperature, 420°; time, 2 hours.

	Catalyser	No Catalyser
Total nitrogen.....	20.6	20.65
Organic base.....	17.4	17.45
Ammonia.....	3.2	3.2

Solution No. 3 = 21.0 cc.  $\frac{N}{10}$  nitrogen; 20 cc.  $H_2SO_4$ ; temperature, 340–365°; time, 3 hours.

	Catalyser			No Catalyser		
Total nitrogen .....	21.0	20.9	21.0	21.75	20.55	20.75
Organic base.....	18.2	17.2	19.1	18.6	18.1	18.95
Ammonia.....	2.8	3.7	1.9	3.15	2.45	1.80

Solution No. 1; 20 cc.  $H_2SO_4$ ; temperature, 380–400°; time, 6 hours.

	Catalyser	No Catalyser
Total nitrogen.....	15.1	15.0 15.1
Organic base.....	9.9	9.25 9.25
Ammonia.....	5.2	5.75 5.85

Same solution; 20 cc.  $H_2SO_4$ ; temperature, 380–400°; time, 7 hours.

	Catalyser	No Catalyser
Total nitrogen.....	14.9	14.9 14.9
Organic base.....	8.1	8.0 9.6
Ammonia.....	6.8	6.9 5.3

These results lead to the conclusion that methylamine is not affected to any considerable extent when digested at low temperature by sulphuric acid with or without catalyser. With rising temperature the amount of ammonia increases.

#### METHYLUREA.

The composition of methylurea suggests that its decomposition products after Kjeldahl digestion might be equal amounts of organic base and ammonia.

Heated  $\frac{1}{2}$  hour in lead bath, temperature about 280°.

Total nitrogen.....	16.0	16.0
Organic base.....	8.0	8.0
	<hr/>	<hr/>
Ammonia.....	8.0	8.0

Heated one hour.

Total nitrogen.....	16.0	16.0
Organic base.....	7.6	7.6
	<hr/>	<hr/>
Ammonia.....	8.4	8.4

Digested by free flame.

$\frac{1}{2}$  hour.

Total nitrogen.....	15.7
Organic base.....	7.3
	<hr/>
Ammonia.....	8.4

1 hour

Total nitrogen.....	15.8
Organic base.....	7.1
	<hr/>
Ammonia.....	8.7

2 hours

Total nitrogen.....	15.7
Organic base.....	5.2
	<hr/>
Ammonia.....	10.5

As shown in these experiments, the maximum (theoretical) amount of organic base can be obtained by short digestion at low temperature. A 30-minute digestion does not change the ratio organic base: ammonia, but long digestion at high temperature will increase the amount of ammonia at the expense of the organic base.

CREATIN.

Since it is possible to determine the organic base group in methyl-urea by appropriate digestion, it was expected that creatin would yield one third of its total nitrogen in form of organic base. Two lines of experiments were carried out. One at a comparatively low temperature, using a lead bath, the other at high temperature by using free flame.

Leadbath: 10 cc. of creatin solution = 21.7 cc.  $\frac{N}{16}$  nitrogen; temperature about 300°.

$\frac{1}{2}$  hour

Total nitrogen.....	11.0	12.1	13.0
Organic base.....	5.4	5.4	5.1

Total nitrogen.....	15.8	15.4	15.1
Organic base.....	5.0	4.75	5.0

1 $\frac{1}{2}$  hours

Total nitrogen.....	16.2	17.5	17.5
Organic base.....	4.6	4.6	4.4

3 hours

Total nitrogen.....	21.5	21.6	21.5
Organic base.....	4.6	4.4	4.5

Free flame: Total nitrogen as determined by Kjeldahl in parenthesis.

10 minutes

Total nitrogen.....	15.25 (20.15)
Organic base.....	3.10

20 minutes

Total nitrogen.....	7.25 (8.0)	7.25 (8.0)
Organic base.....	1.5	1.5

30 minutes

Total nitrogen.....	8.0 (8.0)	8.0
Organic base.....	1.5	1.5

2 hours

Total nitrogen.....	20.15 (20.15)
Organic base.....	3.4

7 hours

Total nitrogen.....	20.10 (20.15)
Organic base.....	1.7

When creatin is digested in a lead bath at a temperature of about 300° for 1 $\frac{1}{2}$ –3 hours it was noticed that the maximum amount of organic base was obtained after  $\frac{1}{2}$  hour's digestion, in spite of the fact that only a part of its nitrogen had been split off. When the digestion is continued, a higher value for the total nitrogen results, but the amount of organic base decreases.

As methylamine showed considerable stability when subjected to the same treatment, it must be assumed that a condensation of methyl groups occurs leading to the formation of dimethylamine. This seems the more evident as dimethylamine was obtained by Engeland<sup>1</sup> as distillation product of creatinin. The digestion at higher temperature facilitates this condensation as shown in the experiments when creatin is digested over the free flame.

The theoretical ratio organic base: ammonia = 1: 3 could not be obtained.

#### CREATININ.

The digestion of creatinin with sulphuric acid and catalyser leads to results similar to those obtained from creatin:

Solution of creatinin, 20 cc. = 25.2 cc. $\frac{N}{10}$ nitrogen; 20 cc. $H_2SO_4$ ; $CuSO_4$ .			
	$\frac{1}{2}$ hour	1 hour	2 hours
Total nitrogen.....	24.8	25.1	24.7
Organic base.....	3.3	2.9	2.0
	<hr/>	<hr/>	<hr/>
Ammonia.....	21.5	22.2	22.7

When Nessler's reagent was added to the filtrate from the mercuric oxide, a dense yellow precipitate resulted, proving the presence of methylamine. To study the effect of high temperature, the substance was digested with the same amount of sulphuric acid after adding the sulphate and sodium phosphate mixture suggested by Folin.<sup>2</sup>

20 cc. $H_2SO_4$ ; 2 grams sulphate mixture; 5 grams sodium phosphate.			
	$\frac{1}{2}$ hour	1 hour	2 hours
Total nitrogen.....	25.1	25.2	25.1
Organic base.....	3.0	1.9	0.3
	<hr/>	<hr/>	<hr/>
Ammonia.....	22.1	23.3	24.8

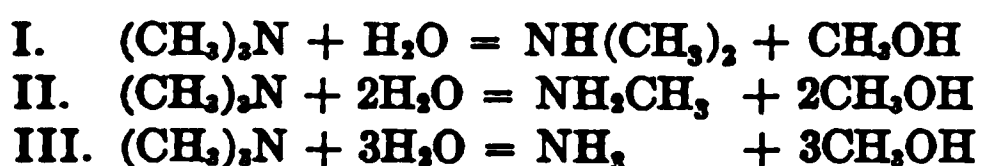
In the filtrate from the mercuric oxide no precipitation, but only slight opalescence occurred when Nessler's reagent was added; hence the organic base was present as secondary amine.

<sup>1</sup> Engeland: *Zeitschr. f. physiol. Chem.*, lvii, p. 65.

<sup>2</sup> Folin: *Zeitschr. f. physiol. Chem.*, xli, p. 241.

## TRIMETHYLAMINE.

When trimethylamine is digested with sulphuric acid and catalyser, three phases of hydrolysis are possible:



According to the amount of water taken up, dimethylamine, monomethylamine or ammonia will result.

This reaction corresponds to the formation of alkylamine as described by Weith,<sup>1</sup> who found that a mixture of mono-, di-, trimethylamine and tetramethylammonium chloride is formed when methyl alcohol and ammonium chloride are heated in a sealed tube for 10 hours at 285°.

In repeating this experiment 3 grams of ammonium chloride and 12 cc. of methyl alcohol were heated in a sealed tube for 4 to 5 hours at 300°.

A similar result was obtained. The formation of mono- and trimethylamine, tetramethylammonium chloride and pyridine could be proven, and there is no reason to suppose that the intermediary secondary product is not also formed. As to the quantity of the quaternary ammonium base it was found that only a small amount was present.

The content of the sealed tube was made up to 250 cc. with distilled water and this solution was used for the following tests.

A fraction gave, with Nessler's reagent, a bright yellow precipitate—indicating the presence of methylamine and the absence of ammonia. Ten cc. were digested with sulphuric acid and catalyser to ascertain the amount of the total nitrogen = 12 cc. Ten cc. distilled with sodium hydrate gave the amount of alkylamine + pyridine (since sodium hydrate does not act upon the tetramethylammonium chloride) = 11.2 cc. The difference in both results is due to the nitrogen of the tetramethylammonium chloride.

Trimethylamine and pyridine were identified by their characteristic odors.

<sup>1</sup> *Ber. d. deutsch. chem. Gesellsch.*, viii, p. 458.

To study the effect of Kjeldahl digestion, a solution No. I of trimethylamine hydrochloride containing 5 grams in 500 cc. water and No. II containing 7.5 grams in 500 cc. water were prepared. Ten cc. of solution I, distilled with sodium hydrate yielded 9.5 cc.  $\frac{N}{16}$  nitrogen; the same result was obtained by the Kjeldahl method, and since Nessler's reagent proved the absence of ammonia, it follows that the total amount of nitrogen was present as alkylamine. A differentiation of the 3 possible amines has not yet been carried out.

10 cc. solution No. I digested with 10 cc. sulphuric acid and copper sulphate for 1 hour (leadbath, low heat)

Total nitrogen.....	9.25	9.25
Organic base.....	9.20	9.20
Ammonia.....	0	0

10 cc., 3 hours (leadbath, low heat)

Total nitrogen.....	9.25	9.25
Organic base.....	9.20	9.20
Ammonia.....	0	0

Solution No. II.

Digested with 20 cc.  $H_2SO_4$  and  $CuSO_4$ , 3 hours (open flame).

	10 cc.	5 cc.
Total nitrogen.....	15.4	7.6
Organic base.....	15.1	7.4
Ammonia.....	0.3	0.2

10cc., 5 hours (highest temperature)

Total nitrogen.....	15.4	15.4
Organic base.....	11.2	11.05
Ammonia.....	4.2	4.35

These data show at once the greater stability of trimethylamine as compared with monomethylamine. A long digestion at high temperature is required to hydrolyze it so far that ammonia can be found among its decomposition products. This must be explained by the following consideration: when trimethylamine is subjected to hydrolysis, the intermediary products will be di- and



monomethylamine; these then are converted into ammonia by further hydrolysis. As is shown in the above determinations, a 3 hours digestion at low heat had caused no change in the alkylamine amount of the substance, though the presence of methylamine could be proven, and the digestion for the same length of time at a higher temperature (open flame) converted only a small fraction of the alkylamine into ammonia.

The results indicate that trimethylamine is more or less completely broken up into mono- and dimethylamine before the decomposition of these products into ammonia begins. This curious phenomenon is being further investigated.

#### PEPTON.

Solution I; 50 grams of "Armour's Pepton," were dissolved in 500 cc. water with small amount of sulphuric acid. Ten cc. of this solution were digested 30 minutes with 20 cc. sulphuric acid and copper sulphate. The distillate contained 61.4 cc.  $\frac{N}{16}$  nitrogen and was diluted to 500 cc.

Separation of organic base and ammonia in 100 cc.

Total nitrogen.....	12.3	12.3
Organic base.....	0.4	0.4
	<hr/>	<hr/>
Ammonia.....	11.9	11.9

The odor of trimethylamine was noticeable. Seven cc. of the original solution were digested with 20 cc. sulfuric acid and copper sulphate for  $\frac{3}{4}$  hour. For the distillate, the total nitrogen was found to be 42.6; it was diluted to 500 cc.

Separation in 100 cc.

Total nitrogen.....	8.5	8.5
Organic base.....	0.2	0.2
	<hr/>	<hr/>
Ammonia.....	8.3	8.3

Also here the odor of trimethylamine was noticed, when 250 cc. were filtered off for the distillation of the organic base.

Solution No. 2 containing 23.1 cc.  $\frac{N}{16}$  nitrogen in ten cc. were digested with 20 cc. sulphuric acid and catalyser for 4 hours.

Total nitrogen.....	23.1 cc.
Organic base.....	0.
<hr/>	
Ammonia.....	23.1 cc.

The same digested for 2½ hours.

Total nitrogen.....	23.1 cc.
Organic base.....	0.
<hr/>	
Ammonia.....	23.1 cc.

When pepton is digested for a short time (½ hour) a small amount of organic base is obtained; after a prolonged digestion this trace disappears.

LECITHIN.

About 1 gram of lecithin was digested with 20 cc. sulphuric acid and copper sulphate for about 1½ hours.

Total nitrogen.....	3.00 cc. $\frac{N}{16}$
Organic base.....	2.6
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Ammonia.....	0.4

The filtrate from the mercuric oxide had the odor of trimethylamine, and when Nessler’s reagent was added it remained colorless, proving the absence of methylamine.

A larger amount was digested for 4 hours. Total nitrogen, 36.2. The distillate was made up to 500 cc. and 100 cc. of this solution were used for the separation of organic base and ammonia.

Total nitrogen.....	7.2	7.2 cc. $\frac{N}{16}$
Organic base.....	5.5	5.5
<hr/>		<hr/>
Ammonia.....	1.7	1.7

The filtrate from the mercuric oxide had strong odor of trimethylamine, and when Nessler’s reagent was added, a dense yellow precipitate was formed indicating the presence of monomethylamine.

There seems to be no doubt that the whole amount of nitrogen as present in lecithin is split off as trimethylamine. This of course will undergo further hydrolysis when the digestion is continued.

#### UREA.

When urea had been digested with sulphuric acid and catalyser, no alkylamine being expected among its decomposition products, it was noticed that in some determinations for the titration of the final distillate a trifle less alkali was used than the theoretical figure. The difference, which had to be explained either as organic base or unabsorbed ammonia, was small, however, not exceeding 0.05–0.1 cc.  $\frac{N}{10}$  acid, and must be considered peculiar to the method of separation.

That the difference is due to unabsorbed ammonia was shown by redistilling the titrated distillate into a solution of Nessler's reagent. The formation of oxydimercurammonium iodide, noticeable by the color of the distillate, identified the volatile nitrogen compound as ammonia. Almost the same difference was obtained in some cases where larger amount of substance had been digested.

Digested by free flame  $\frac{1}{2}$  hour.

Total nitrogen.....	16.	16.
2nd distillation.....	0.06	0.06
	<hr/>	<hr/>
Ammonia.....	16.0	16.0

Larger amount digested by free flame,  $\frac{1}{2}$  hour.

Total nitrogen .....	48.7
2nd distillation .....	0.05

#### URIC ACID.

A solution of uric acid was used containing 9.2 cc.  $\frac{N}{10}$  nitrogen in 10 cc.

Digestion by free flame.  
 $\frac{1}{2}$  hour

Total nitrogen.....	9.1
2nd distillation .....	0.0
	<hr/>
Ammonia .....	9.1

1 hour	
Total nitrogen .....	9.1
2nd distillation .....	0.0
<hr/>	
Ammonia .....	9.1

2 hours	
Total nitrogen.....	9.2
2nd distillation .....	0.05
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Ammonia.....	9.2

About 0.25 gm. digested $\frac{1}{2}$ hour.	
Total nitrogen .....	58.3
2nd distillation .....	0.05

As in the case of urea—the total nitrogen of uric acid is present as ammonia after Kjeldahl digestion.

GLYCOCOLL.

Digested by free flame.		
1 hour		
Total nitrogen.....	9.25	9.25
2nd distillation.....	0.05	0.

Also here a larger amount of substance showed the absence of alkylamine.

Total nitrogen.....	45.6
2nd distillation .....	0.06

When digested according to Kjeldahl the nitrogen of glycocoll is present as ammonia.

HIPPURIC ACID.

Since hippuric acid decomposes in forming benzoic acid and glycocoll, analogous results were to be expected:

Digestion by free flame:	
$\frac{1}{2}$ hour	
Total nitrogen .....	19.5
2nd distillation .....	0.00

1 hour

Total nitrogen .....	19.7
2nd distillation .....	0.05

0.85 gr. were digested with 20 cc.  $\text{H}_2\text{SO}_4$  and  $\text{CuSO}_4$  for  $\frac{1}{2}$  hour.

Total nitrogen .....	46.1
2nd distillation .....	0.05

These data do not confirm Folin's<sup>1</sup> observation that methyl or alkylamines are obtainable from ordinary amino acids, but indicate that the isonitril reaction he obtained from glycocoll or hippuric acid is due to impurities.

#### SUMMARY.

Considering the structures of the different substances which have been subject to separation after Kjeldahl digestion, it must be concluded that mono-, di- or tri-methylamine can be obtained when the nitrogen compound contains the group  $\text{CH}_3\text{—N=}$ ,  $\text{CH}_3\text{—NH—}$  or  $(\text{CH}_3)_3\text{N=}$ .

The method described in this paper can probably be used as a general qualitative method for determining in nitrogen-containing substances the presence of alkylamine groups, and can probably also be used for approximately quantitative determinations as well.

This research was carried out at the suggestion of Doctor Folin, to whom I feel indebted for his interest and advice.

<sup>1</sup> This *Journal*, iii, p. 84, 1907.



# ON THE ALLEGED OCCURRENCE OF TRIMETHYLAMINE IN URINE

By C. C. ERDMANN.

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(Received for publication, April 25, 1910.)

In 1856, Dessaignes<sup>1</sup> pointed out the occurrence of trimethylamine in urine, separating from 65 liters, 17 grams of the platinum double salt, equal to 3.7 grams of trimethylamine. He leaves it doubtful, however, whether trimethylamine is to be considered a preformed constituent of urine or a decomposition product.

Filippo de Filippi<sup>2</sup> does not attempt to solve this question, but merely carries out the determination of trimethylamine in urine by liberating the alkylamines by distillation with sodium hydrate and subsequent isolation of the tertiary organic base, coming to the conclusion that trimethylamine is a normal product of metabolism.

Dessaignes' idea was taken up by Takeda,<sup>3</sup> who endeavored to set free the trimethylamine by using a less radical method in order to spare nitrogen compounds which might yield trimethylamine when digested according to Kjeldahl, or even when only distilled with strong alkali. In order to carry out the determination of trimethylamine under such conditions, he used a modification of the Krüger-Reich apparatus, distilling the urine after addition of magnesium oxide in a vacuum of about 35 mm. at a temperature of about 40°. Applying this method to several specimens of fresh normal urine, he was unable to precipitate any trimethylamine in the distillate by means of gold chloride; while in specimens which had stood for from 24 to 36 hours (decomposition probable), small

<sup>1</sup> *Annalen*, c, p. 218.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, xlix, p. 433.

<sup>3</sup> *Arch. f. d. ges. Physiol.*, cxxix, p. 82.

## 58 Occurrence of Trimethylamine in Urine

amounts of trimethylamine were obtained. Using a method had been suggested by Folin<sup>1</sup> for the determination of ammonia in urine (distillation under atmospheric pressure with magnesia oxide), in every case trimethylamine was obtained showing the latter method is the more radical one. The same alkali used in both cases, the higher temperature of the Folin process must be responsible for the decomposition of nitrogen compounds yielding trimethylamine. The effect of different alkalies is expressed in one of Takeda's experiments<sup>2</sup> consisting of three distillations under different conditions:

- |  |  |
|--|--|
| 1. His own method (Mg O)               | = no trimethylamine.                       |
| 2. Folin's method (Mg O)               | = a small amount.                          |
| 3. Filippo de Filippi's method (Na OH) | = a considerable amount of trimethylamine. |

Filippo de Filippi's method consists in distilling a whole 24 specimen or a large part thereof by steam, after the addition of 10–15 grams of potassium hydrate. The distillate is received in hydrochloric acid and evaporated. The pulverized residue is repeatedly extracted by alcohol to remove the ammonium chloride and the final residue containing the alkylamines and traces of ammonia is dissolved in a little water and introduced into an apparatus of special construction. The separation of trimethylamine from the mono-, dimethylamine and ammonia is effected by sodium hypobromide, which destroys the ammonium chloride as well as mono- and dimethylamine. This method seems to be radical enough for the determination of the total trimethylamine but it is too radical for the determination of the preformed.

The volatile character of trimethylamine suggested the adoption of Folin's new method<sup>3</sup> for the determination of ammonia in urine: the ammonia is set free by sodium carbonate plus sodium chloride, and a strong air current is used to carry the ammonia into the  $\frac{N}{10}$  acid. The results obtained for ammonia by this method are constant even when the passing of the air current is continued for a much longer time than is required to carry over the ammonia. The same apparatus and the same alkali were used for the

<sup>1</sup> *Zeitschr. f. physiol. Chem.*, xxxii, p. 515.

<sup>2</sup> *Arch. f. d. ges. Physiol.*, cxxix, p. 86.

<sup>3</sup> *Zeitschr. f. physiol. Chem.*, xxxvii, p. 161.



mation of trimethylamine,—the only difference being that suction was substituted for the air-blast, thus allowing a larger number of determinations to be carried out at the same time and reducing the possibility of loss by leakage.

A solution of trimethylamine hydrochloride (free from ammonia) was prepared,—50 cc. of the solution containing 7.65 cc.  $\frac{N}{10}$  nitrogen. After the addition of 2 grams of sodium carbonate and 8 grams of sodium chloride, a strong air current was passed through for different periods in order to find out the shortest time in which trimethylamine will be carried over.

	Air passed for 6 hours	2 hours
Trimethylamine	7.65 cc.	7.65 cc.
50cc. = 7.65cc. $\frac{N}{10}$ N	7.70 cc.	7.70 cc.

A solution of trimethylamine sulphate led to the following results:

	Air passed for 1 hour	1½ hours
Trimethylamine	5.25 cc.	5.95 cc.
50cc. = 6.0cc. $\frac{N}{10}$ N	5.75 <sup>1</sup> cc.	6.00 cc.

As shown in the above experiments, a strong air current applied for from 1½ to 2 hours will carry over the trimethylamine quantitatively.

The object of this investigation was to determine whether pre-formed trimethylamine is to be found in normal urine,—no quantitative estimation being aimed at. For this, a qualitative test of great delicacy was relied upon: the characteristic odor of trimethylamine, which according to Kaufmann<sup>2</sup> can be detected in solution 1: 2000000.

Application of the method to fresh urine: 24 hours specimens of normal persons (five male and five female nurses of the hospital) on normal diet were collected. Fifty cc. of urines, 3–4 grams of sodium carbonate and 10–15 grams of sodium chloride were used for each determination, air being passed for 2–2½ hours.

<sup>1</sup>The difference in the parallel determination is due to the different diameter of the cylinders which received the trimethylamine solution. The one with smaller diameter offered a higher column of liquid to the air current.

<sup>2</sup>*Neurolog. Centralblatt*, vi, p. 261. 1908.

## 60 Occurrence of Trimethylamine in Urine

Males	Amount	Weight.	Total nitrogen.
A.	1000	205 lbs.	17.1 cc.
B.	1700	150½	9.5 cc.
C.	1500	145½	6.5 cc.
D.	1300	165	6.2 cc.
E.	1900	170	24.0 cc.
Females	Amount	Weight.	Total nitrogen.
A.	900	162 lbs.	17.5 cc.
B.	2000	137	5.2 cc.
C.	800	124½	9.6 cc.
D.	800	142½	15.9 cc.
E.	1100	169	18.65 cc.

When the amount of the nitrogen had been ascertained by titration the separation of the ammonia from the alkylamine was effected. It was found that either there was no organic base present, or very small quantities only.

The odor of trimethylamine could not be detected in any of these cases. Although trimethylamine can be obtained from any urine after Kjeldahl digestion,—by distillation of urine by strong alkali,—or even by using the Folin method on urine which has stood for any length of time, it must be concluded that fresh, normal urine does not contain trimethylamine.

<sup>1</sup> This *Journal*, viii, p. 41, 1910.

## THE STUDY OF AUTOLYSIS BY PHYSICO-CHEMICAL METHODS. II.

By ROBERT L. BENSON AND H. GIDEON WELLS.

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(Received for publication, May 17, 1910.)

In previous papers<sup>1</sup> were reported the results of studies on the influence of the thyroid and kidney on post mortem autolysis of liver tissue at body temperature. These experiments were carried on by different methods; (1) by ascertaining the proportion of nitrogen which existed in different forms, (a) coagulable, (b) non-coagulable but precipitable by zinc sulphate in acid solution, (c) neither coagulable nor precipitable by zinc sulphate; (2) by determining the amount of depression of the freezing point; (3) by the rise in the electrical conductivity of the autolyzing mixture. The experiments as carried out failed to show that either thyroid or kidney extracts have any influence upon the rate of autolysis of liver tissue *in vitro*. Finding that the physico-chemical methods offered certain exceptional advantages as a means of study of autolysis, as contrasted with methods in common use, we have applied them in a number of experiments in order to become more familiar with their value and limitations; incidentally, observations have been made upon the rate of autolysis in different tissues, the availability of different antiseptics for the study of autolysis by physico-chemical methods, and the inhibitory action of blood serum upon autolytic processes.

### INFLUENCE OF ANTISEPTICS UPON AUTOLYSIS.

It is generally understood that the rate of autolysis is more or less hindered by any antiseptic that may be added and that the ideal method is aseptic autolysis. Difficult or impossible as it is

<sup>1</sup> Wells: *Amer. Journ. of Physiol.*, xi, p. 351, 1904. Wells and Benson: *this Journal*, iii, p. 35, 1907.

to keep autolyzing substances free from bacterial growths for any length of time without the use of antiseptics, the difficulties are greatly increased when the autolysis is to be measured by physical methods, especially when it is desired to carry on the autolysis in a conductivity cell. Therefore it is necessary to use antiseptics in practically all experiments, and we first tried various antiseptics to test their availability. Toluene, which is most commonly used, is said by some not to be altogether reliable as a bactericidal agent, but there is usually no difficulty in preventing bacterial growth in fluids if they are shaken thoroughly with the toluene and then a layer of toluene kept on the top. Vandeveldel summarizes the results of his investigations on this point as follows: Formalin destroys bacteria but prevents enzyme action, and the effect of phenol and alcohol is quite similar. Thymol is inefficient at body temperature. Toluene permits enzyme action but it is not altogether reliable as a bactericidal agent. Yoshimoto<sup>2</sup> finds that within certain concentrations boric acid and salicylic acid increase the rate of autolysis as compared with chloroform, which we may believe is because of their acidity, since all acids in proper concentrations favor autolysis.<sup>3</sup> Under the most favorable concentration the amount of conversion of the nitrogen of the liver into soluble form with several antiseptics in an autolysis of seventy-two hours duration, was as follows:

Chloroform water, 21.6 per cent; alcohol in 5 per cent solution, 32 per cent; mustard oil in one-eighth saturated solution 39 per cent; boric acid in 1 per cent solution, 40.8 per cent; salicylic acid in one-half saturated solution, 47.4 per cent.

Although salicylic and boric acids seem to be favorable antiseptics for autolysis experiments, yet the amount of crystalloidal substance introduced by the solutions of such concentration is so great as to obscure somewhat the relatively small effect on the freezing point and conductivity that is produced by autolysis; furthermore, the rate of autolysis in acid solution is not a fair measure of autolytic activity, in view of the great influence which weak acids have upon autolysis. For the purpose of physical

<sup>1</sup> Vandevelde: *Biochem. Zeitschr.*, iii, p. 315, 1907.

<sup>2</sup> Yoshimoto: *Zeitschr. f. physiol. Chem.*, lviii, p. 341, 1908.

<sup>3</sup> See Arinkin: *Zeitschr. f. physiol. Chem.*, liv, p. 192, 1907.

asurements it is preferable to use an antiseptic which does not  
ize extensively and which dissolves to but a slight degree in  
er. Toluene, chloroform and thymol meet these requirements,  
if an excess of the antiseptic is always present the solution

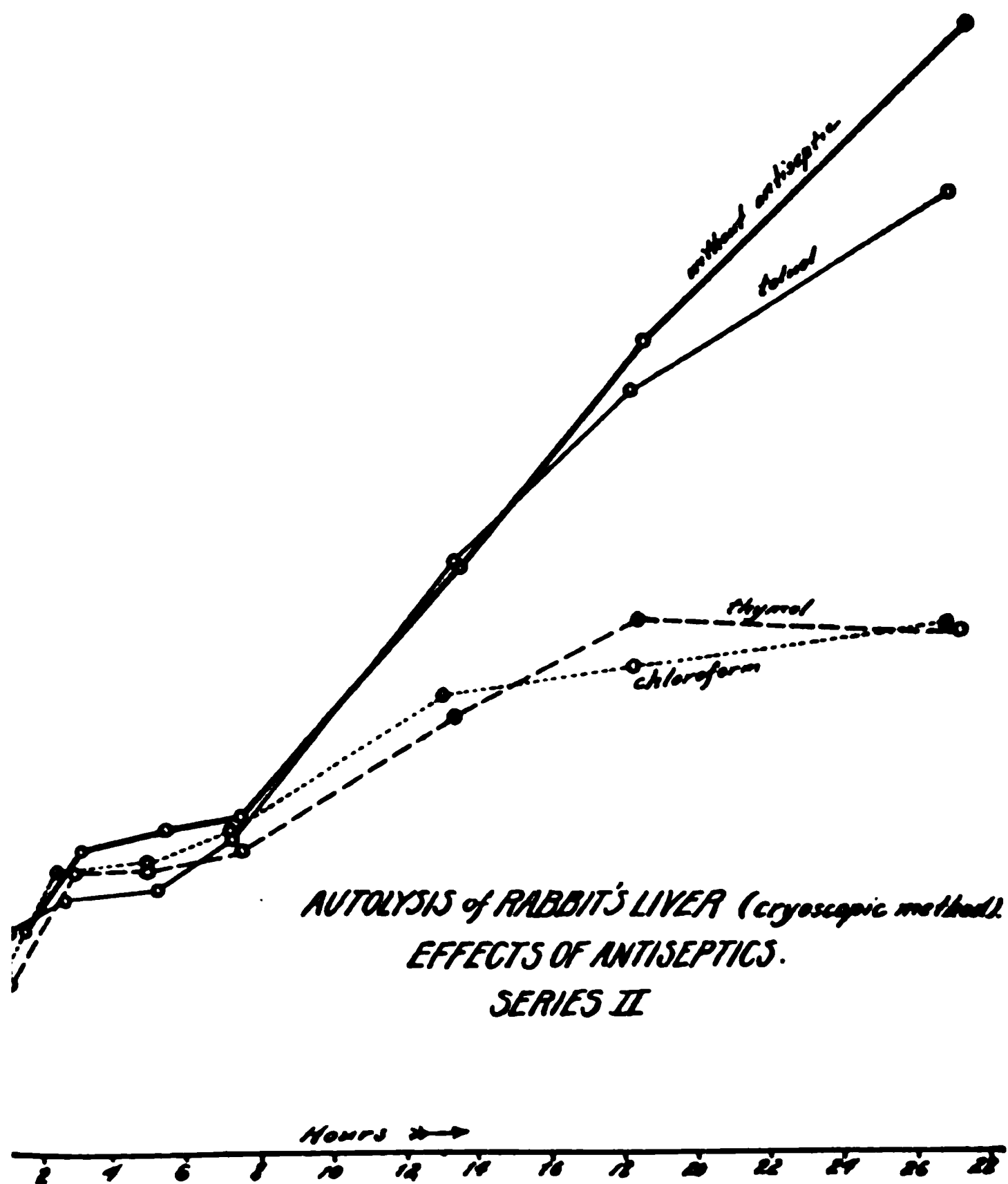


FIG. I.

ains saturated and so there is no change in concentration pro-  
ed by evaporation. Several experiments were performed, all  
which indicate that toluene depresses the autolysis less than the

other two antiseptics mentioned,<sup>1</sup> an extreme case being shown by the accompanying diagram of the curve obtained in one experiment (Fig. I). We found that the best results were obtained if water was saturated with toluene at the temperature at which the experiment was to be conducted, some time before adding it to the tissue that was to be autolyzed. Toluene possesses one great advantage over chloroform in that the excess toluene covers the surface and prevents any changes in concentration of the underlying fluid from evaporation, which might somewhat modify the freezing point and conductivity.

#### THE APPLICABILITY OF PHYSICAL METHODS.

Probably the first to study enzyme action by the conductivity method was Sjöqvist<sup>2</sup> who found that the hydrolysis of albumins by pepsin was attended by a decrease in conductivity. Since his work appeared the method has been only occasionally used. The freezing point offers what is probably an even better method of obtaining a long and full series of readings on autolysis, and was first employed by Sabbatini,<sup>3</sup> who studied the freezing point of various animal tissues, but he did not study autolyzing tissues. Fredericq,<sup>4</sup> Delrez<sup>5</sup> and Liagre<sup>6</sup> have used the cryoscopic method for studying the rate of autolysis. Its advantage over the conductivity method is that it records the presence of non-electrolytes as well as of electrolytes, and affords an accurate measure of the total number of ions and molecules in the solution, while the con-

<sup>1</sup> R. Chiari: (*Arch. f. exp. Path. u. Pharm.*, lx, p. 256, 1909) claims that chloroform, alcohol and ether hasten the onset of autolysis by dissolving the cell lipoids, thus destroying cellular membranes and permitting the enzymes to have access to the protoplasm. This view is not in harmony with the observations we have made, namely, that chloroform and toluene depress autolysis, which is entirely in agreement with the experience of others who have made comparative studies of aseptic and antiseptic autolysis. It is impossible to tell, from Chiari's paper, whether his observations were controlled by suitable experiments, but no mention is made of such controls.

<sup>2</sup> Sjöqvist: *Skand. Arch. f. physiol.*, v, p. 364, 1895.

<sup>3</sup> Sabbatini: *Arch. ital. de biol.*, xxxvi, p. 440, 1901.

<sup>4</sup> Fredericq: *Bull. acad. méd. belg.*, Nov. 29, 1902.

<sup>5</sup> Delrez: *Arch. internat. physiol.*, i, p. 159, 1904.

<sup>6</sup> Liagre: *ibid.*, p. 172.

ductivity of the autolyzing solution is an index of the number of ions only. Consequently, when the enormous protein molecule breaks down into a number of simpler molecules the freezing point depression is increased in exactly that ratio, it making little difference whether the products of the hydrolysis are electrolytes or non-electrolytes. This gives the cryoscopic method a decided advantage over the conductivity method, for many of the products of protein hydrolysis, especially some of the mono-amino acids, have extremely low conductivities. On the other hand, comparison of the results obtained by the two methods may throw some light upon the nature of the substances that have been formed during autolysis. For instance, the conductivity does not record the carbohydrates and fats; hence, if in a given specimen the curves for autolysis by both the conductivity and the cryoscopic method are parallel, the increase is presumably due to an increase in the amount of dissolved electrolytes.

The conductivity of the isolated products of proteolysis in aqueous solution has been investigated by Walter Neumann<sup>1</sup> in the case of the products of tryptic digestion. Peptone he found to be strongly acid, and therefore to conduct well. Diamino acids conduct well, because of their basic character, as likewise do the strongly acid diatomic mono-amino acids (aspartic and glutamic acids.) The other amino acids, such as glycocoll and leucine, have an extremely low conductivity. Bayliss<sup>2</sup>, whose results with the isolated amino acids agree with those obtained by Neumann, found that the combined effect of the peptones and diamino acids formed in tryptic digestion was sufficient to overshadow greatly the increase in conductivity due to the splitting off of inorganic salts from the protein. He also found that changes of internal friction are insufficient to account for the increase in conductivity which develops during tryptic digestion. Gelatin produces a decrease in the conductivity of potassium chloride equal to 3.4 per cent of the original conductivity for each 1 per cent of gelatin added, and caseinogen decreases it 2.7 per cent; but sugar, a non-colloidal non-electrolyte, also produces a decrease of 2.8 per cent in conductivity for each 1 per cent of sugar added; therefore,

<sup>1</sup> Neumann: *Zeitschr. f. physiol. Chem.*, xlv, p. 216, 1905.

<sup>2</sup> Bayliss: *Journ. of Physiol.*, xxxvi, p. 221, 1907.

the two effects are in the same order of magnitude. Moreover, gradually warming a gelatin solution so as to destroy the “gel” condition does not produce a sudden change in the conductivity. During tryptic digestion the change in conductivity is 100 per cent or more, and hence much more than can be accounted for by change in viscosity, and must be due to the products of protein cleavage.

Jackson and Pearce<sup>1</sup> found that the proportion of nitrogen in different forms in the incoagulable nitrogen of dog’s liver changes during autolysis, as follows:

	NORMAL LIVER	AUTOLYSED LIVER
Non-coagulable nitrogen.....	9.7	29.1
Diamino acids.....	15.9	19.0
Monoamino acids.....	6.7	23.9

If previous to autolysis the liver was irrigated with Ringer’s solution, to remove the serum which inhibits autolysis, the increase in mono-amino acid nitrogen they found to be even greater. These figures show another reason why the cryoscopic method gives a better insight into autolytic changes than the conductivity method, for during autolysis the greatest increase is in the mono-amino acids which are for the most part poor conductors. On the other hand, this defect is offset to a greater or less degree by the fact that in autolysis, in contrast to tryptic digestion, there is extensive deamidization of amino acids and purines by amidases, and this results in the formation of ammonia and free organic acids or their salts, which are all good conductors.

The accompanying curves (Fig. II) illustrate the differences in the effects of autolysis upon the freezing point and the conductivity. In this experiment a portion of dog liver was combusted and the freezing point and conductivity of the ash were determined. Both are plotted as one base line, and the freezing point and conductivity of a sample of the same liver undergoing autolysis plotted on the same scale as the ash. The two curves show roughly that autolysis affects the freezing point much more than it affects the conductivity, this being due to the fact that many of the products of autolysis have a relatively low conductivity, and yet being soluble, exert their full influence on the freezing point.

<sup>1</sup> Jackson and Pearce: *Journ. of Exp. Med.*, ix, p. 520, 1907.



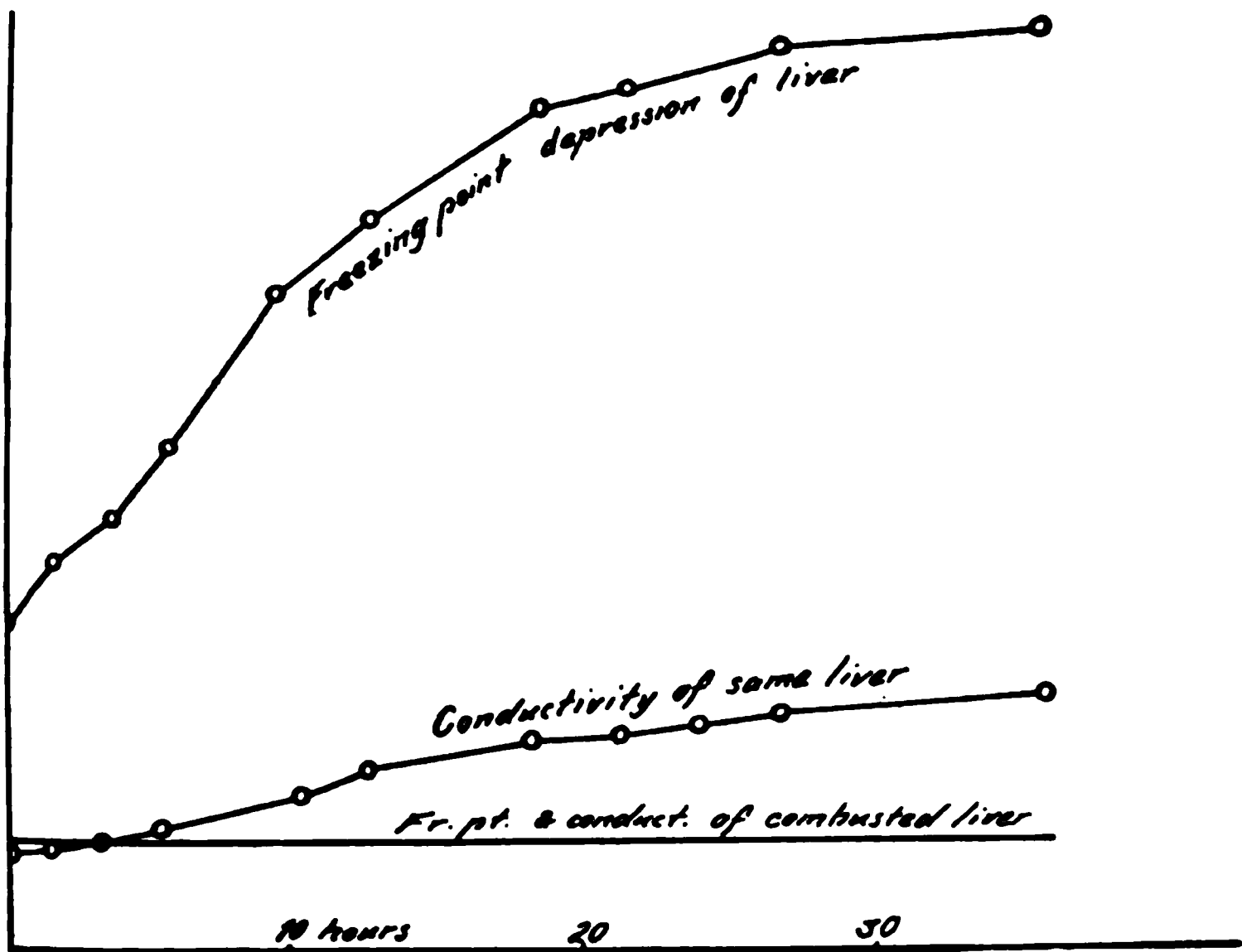


FIG. II.

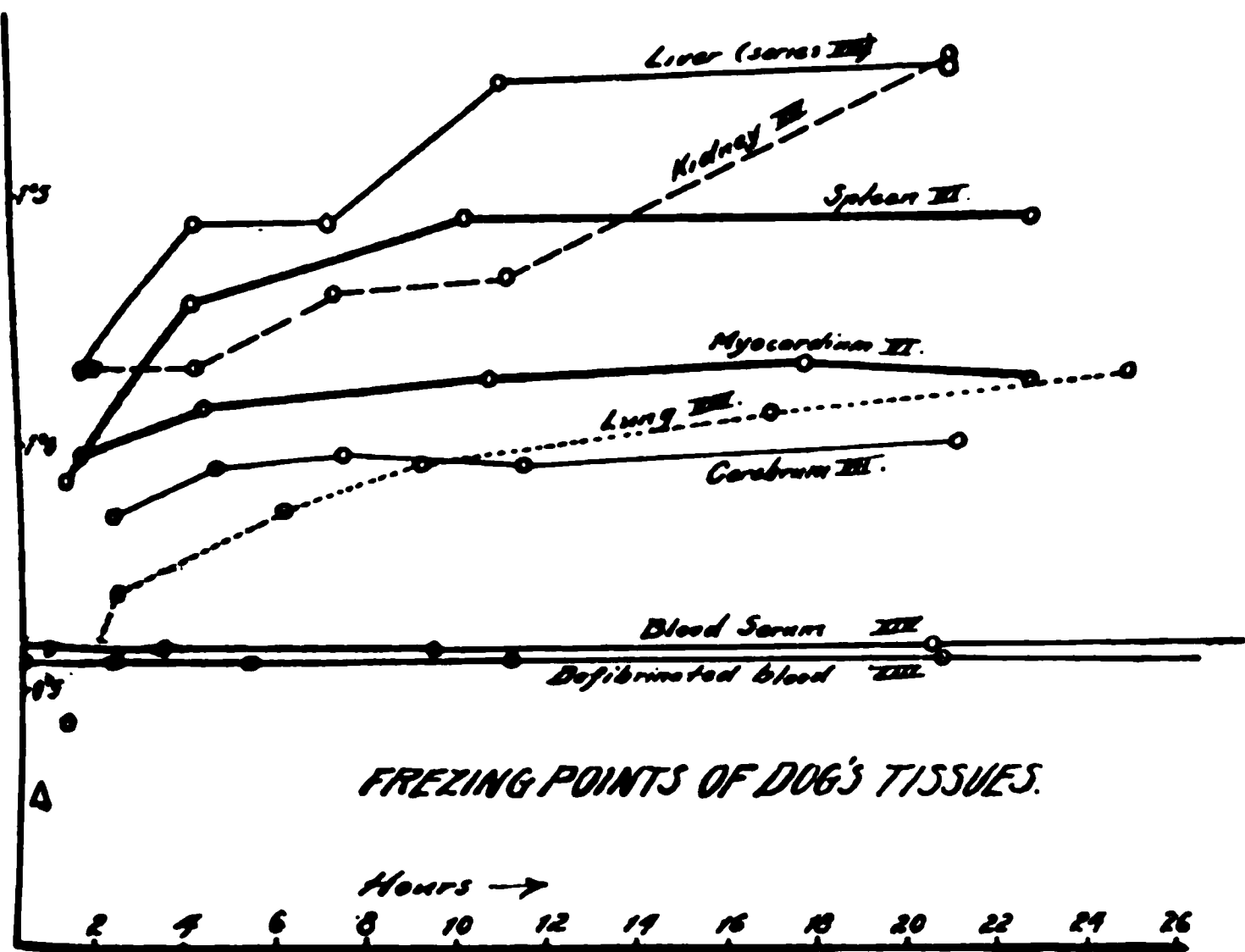


FIG. III.

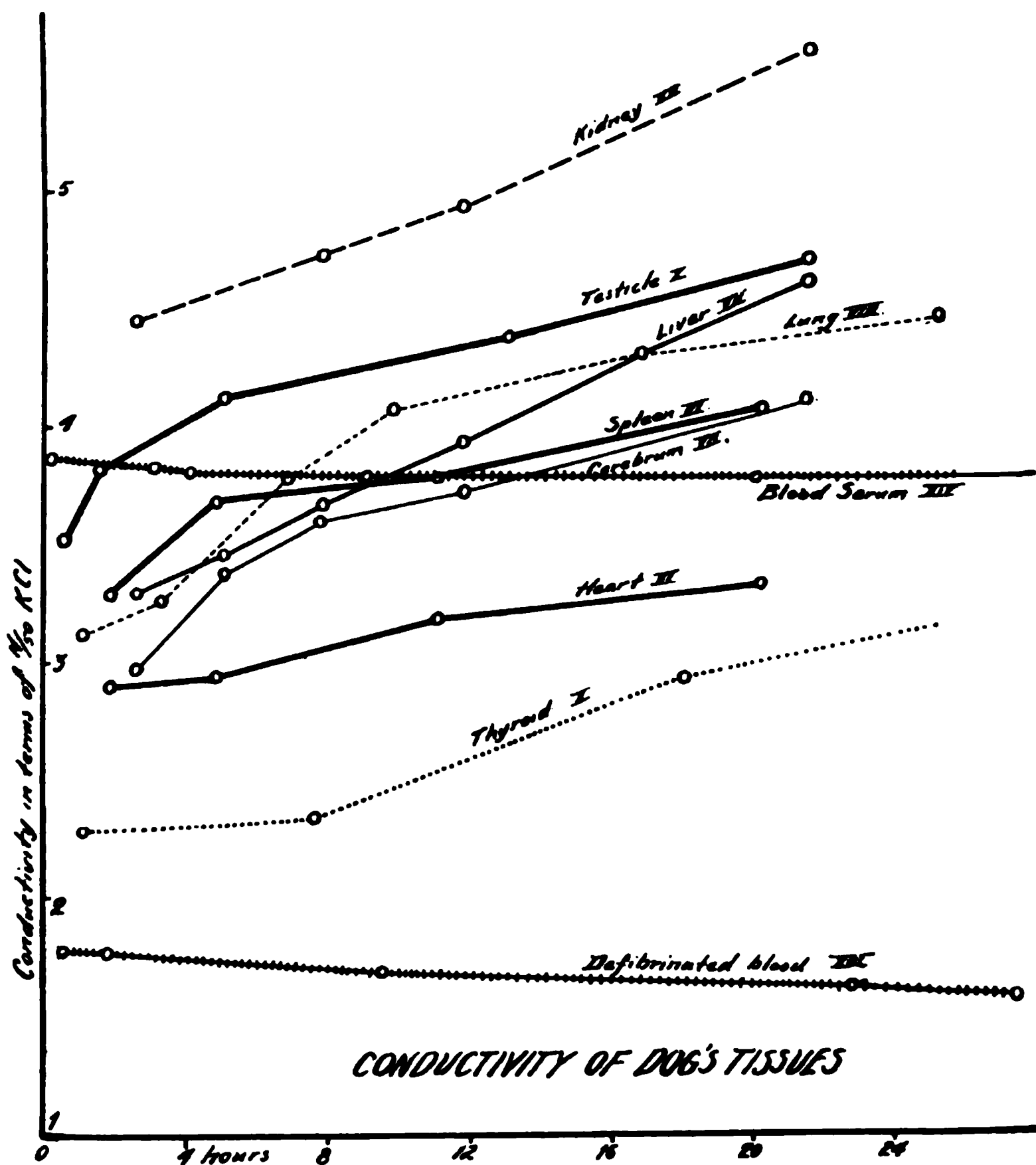


FIG. IV.

Consideration of the curves in Figures III and IV, which have been plotted from several different series of autolyzing organs, also shows the differences in the form of the curves of freezing point and conductivity changes. It will be particularly noticed that in the case of the freezing point the most rapid and marked changes occur before the twelfth hour, after which time the changes are in most cases very slight. The conductivity, however, which rises relatively little in the first twelve hours, continues to rise steadily to the twenty-fourth hour, making the curve approach

more nearly a straight line. This fact indicates that at first the newly formed substances are chiefly non-conductors, but that later the new molecules formed are largely electrolytes. Probably the first, comparatively sudden, rise in both conductivity and freezing point depends upon the escape of the cell contents from the tissues and their solution in the fluid. This is followed by a rise due to the formation of sugar from glycogen, fatty acids and glycerin from the fats, proteoses, peptones and amino acids from the proteins, and nucleic acid and free purines from the nucleoproteins; these substances are, for the most part, poor conductors, and hence the rise in the conductivity is relatively less than the rise in freezing point depression. Later there is introduced the deamidization of the amino acids and purines, which leads to the formation of ammonia from the amino groups, and organic acids from the amino acids, and as at this time the rate of disintegration of the large molecules has greatly slowed down we find the freezing point changing slowly while the conductivity continues to increase.

Again comparing the two sets of curves we find that there are certain differences between different organs. The kidney has a high initial conductivity compared with the other tissues, presumably because of the large amount of urinary salts present in its tubules; but its initial freezing point is the same as that of the liver, indicating that the liver is relatively rich in soluble non-electrolytes, which agrees well with our knowledge of the chemistry of these organs. The lung seems to be very poor in soluble materials, and such as there are must be electrolytes, for the  $\Delta$  is relatively much lower than the conductivity; however, the rate of autolysis is about the same as that of the liver or kidney. Brain tissue shows relatively more increase in conductivity than in freezing point, indicating that the rate of autolysis is low, but that the molecules formed are mostly good conductors; presumably this is because the protein decomposition in autolyzing brain is relatively small, and the new molecules are chiefly such substances as cholin and fatty acids, which conduct relatively well. On the whole the rate of autolytic change, as measured by the amount of rise between the initial and final readings is about the same in the different organs whether measured by the cryoscopic or the conductivity methods, the liver and kidney leading, closely followed by the lung and spleen, while autolysis of brain and myocar-

dium is much slower. These results are much the same as those obtained by chemical analysis or by observation of the microscopical changes in different organs and tissues.<sup>1</sup>

The fact that the relative effects of autolytic changes on conductivity and freezing point are comparable with different organs, indicates that the results obtained by conductivity measurements have nearly as much value in comparative studies of the rate of autolysis as have the cryoscopic measurements, in spite of the fact that the latter give a more exact picture of the total change that is taking place in the tissue. Since studies of autolysis are in most cases comparative, the conductivity method can, therefore, often be used to advantage. This is particularly the case when the amount of material available is small, as, for example, in studying autolysis in a single small organ, such as the thyroid or adrenal. The autolysis may be conducted with one 10 c.c. sample in a small conductivity cell, and the readings made as often as desirable with the same sample. Freezing point determinations, however, should be made when possible on separate samples of 10 to 20 c.c. and hence a considerable amount of material must be available in order to secure a series of any considerable extent.

That the freezing point and conductivity measurements actually represent changes that are taking place in the tissues, and are not seriously altered by errors in technic due to the nature of the materials and methods, is well demonstrated by the curves obtained with blood serum and blood (see Figs. III and IV). Repeated readings of the freezing point show that these fluids undergo no appreciable change during twenty-four hours, and even much longer. The conductivity of the blood, indeed seems to fall slightly, the amount becoming quite appreciable if readings are taken for several days. Stewart<sup>2</sup> observed this same phenomenon inconstantly in his experiments, in which precautions to avoid bacterial contaminations were not taken. In our experiments, which were controlled culturally, the decrease in conductivity was observed constantly. Stewart explains this change as due to laking of the blood; the hemoglobin which enters the solution then increases the internal friction, thus

<sup>1</sup> Wells: *Journ. of Med. Research*, xv, p. 149, 1906.

<sup>2</sup> Stewart: *Journ. of Physiol.*, xxiv, p. 211, 1899.

reducing the conductivity. Possibly the hemoglobin also depresses conductivity by combining with the free ions. That hemoglobin is probably the cause of the decreased conductivity we have demonstrated by adding to dog serum pure hemoglobin, freshly prepared from dog's blood, and found that it decreased the conductivity considerably, as Stewart had previously shown. The decrease in conductivity which Sjöqvist observed during digestion of protein by pepsin-HCl, is probably also produced in a similar way by the proteins, proteoses and peptones combining with the free ions in the solution, and by the increased internal friction of the augmented number of colloidal molecules.

#### THE INFLUENCE OF SERUM UPON AUTOLYSIS.

In addition to blood and serum, cerebro-spinal fluid and thoracic duct lymph of the dog have been examined, and found to remain without any appreciable change in the freezing point or conductivity when left standing at 36.8 per cent under toluene for two days. It cannot be said whether this absence of autolysis in these fluids depends upon absence of autolytic enzymes or upon their inhibition by the anti-bodies the fluids contain.

That the serum of animals has a strong inhibiting influence upon autolysis is well known, and this effect is similar to its inhibiting action on tryptic digestion which was first shown by Hahn.<sup>1</sup> Serum inhibits to a greater or less degree the autolysis of the fixed tissue elements, as shown by Baer and Loeb and others<sup>2</sup> and also autolysis and heterolysis by leucocytes.<sup>3</sup>

This inhibitory effect can be demonstrated well by conductivity and freezing point determinations, as shown in Fig. V. In these curves it is seen that during twenty-four hours there has been practically no change of conductivity in the liver emulsion in serum, although in water the increase in conductivity is marked, and what is particularly striking in this experiment is the persistence of the

<sup>1</sup> Hahn: *Berl. klin. Woch.*, xxxiv, p. 499, 1897.

<sup>2</sup> Baer and Loeb: *Arch. f. exp. Path. u. Pharm.*, liii, p. 1, 1905; Baer: *ibid.*, lvi, p. 68, 1906; Hildebrandt: Hofmeister's *Beiträge*, v, p. 463, 1904; Wells: *Journ. of Med. Research*, xv, p. 149, 1906.

<sup>3</sup> Opie: *Journ. of Exp. Med.*, vii, p. 316, 1905; Opie and Barker: *ibid.*, ix, p. 207, 1907.

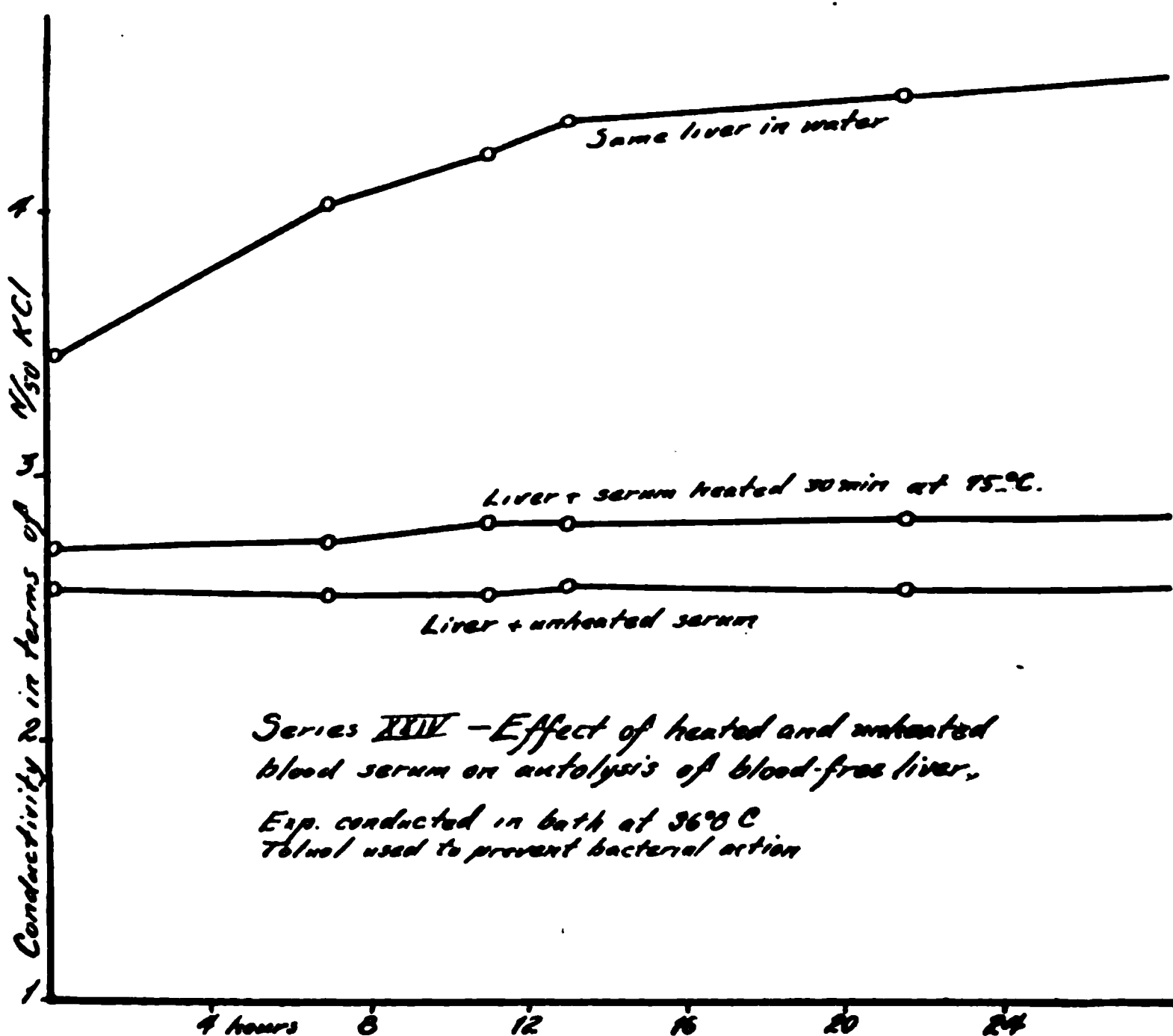


FIG. V.

inhibition in spite of heating of the serum to  $75^{\circ}$  for thirty minutes. Opie found that the inhibiting action of serum upon autolysis of leucocytes is destroyed by heating thirty minutes at  $75^{\circ}$ , but not at  $70^{\circ}$ . Our results all indicate almost complete inhibition of the autolysis of dog liver by dog serum.<sup>1</sup> Heating serum diluted with an equal volume of distilled water to  $60^{\circ}$  or  $70^{\circ}$  for one half-hour produces no visible change in the serum, but if heated at  $75^{\circ}$  or  $80^{\circ}$  it becomes more or less cloudy or even jelly-like; such jellied serum, however, retains, either unimpaired or but slightly reduced, its power to inhibit autolysis. Even serum diluted with four volumes of water and heated thirty minutes at  $85^{\circ}$  and  $95^{\circ}$ , when added to

<sup>1</sup> Schryver (*Biochem. Journ.*, i, p. 144, 1906) has found that this inhibition is not specific, as serum from one species of animals inhibits the autolysis of tissues of other species, and Opie and Barker observed the same lack of specificity in the inhibition of leucocytic protease by serum.

liver emulsion (400 c.c. diluted serum to 67 c.c. of 33 per cent liver emulsion) was found either entirely to prevent appreciable changes in the freezing point of the emulsion, or to limit the change to a small fraction of the change taking place in liver emulsion without serum. This thermostable character of the inhibiting action of serum is in harmony with the results which were obtained by Baer and Loeb; they found that liver autolysis was inhibited by serum which had been heated to boiling, almost as effectively as by fresh serum. This inhibitive property was found to reside chiefly in the serum albumin, which, like the serum, inhibits autolysis equally well after being heated. Serum globulin, when isolated and free from albumin seems rather to accelerate autolysis, but this effect is destroyed by heat. The entire inhibiting effect of serum, however, is not accounted for by these proteins, and Baer believes that other unknown factors must enter into reaction. Although the anti-leucoprotease of Opie also is found to reside in the albumin fraction of the serum, its thermolabile property indicates that it is entirely distinct from the thermostable substance which inhibits autolysis of the fixed tissue cells. The so-called "antitrypsin" of normal serum is also heat resistant.<sup>1</sup> In connection with the subject of inhibition of intracellular proteolytic enzymes by serum, it is of interest to observe that a specific intracellular oxidizing enzyme, uricase, seems not to be inhibited by serum.<sup>2</sup> Similarly Baer has found that deamidization can go on in the presence of serum which inhibits earlier stages of proteolytic disintegration, but he did not ascertain whether the new-formed ammonia was derived from amino acids or purines. This fact may be taken as evidence in favor of the view that autolysis does not take place in normal cells under normal conditions, since known normal intracellular enzymes are here found not to be inhibited by serum, while the intracellular enzymes of protein destruction are held in check by normal serum.

Longcope, studying the histological changes in autolyzing liver tissue, found that the inhibition of nuclear and cytoplasmic disintegration which is exerted by serum, is not impaired by first heating the serum at 65° for fifty minutes. When heated at 85° for

<sup>1</sup> See Bauer: *Zeitschr. f. Immunität.*, v, p. 186, 1910.

<sup>2</sup> Wells and Corper: *this Journal*, vi, p. 321, 1909.

twenty or thirty minutes, however, the inhibitory effect was greatly reduced as contrasted with unheated serum; no comparison of the heated serum with salt solution is mentioned. Wells, who studied the histological changes produced by autolysis in the kidney, found that in the presence of serum the rate of nuclear disintegration was reduced to about half the rate observed in kidney tissue kept in salt solution, while the disintegration of the cytoplasm was almost entirely inhibited by the serum. This fact suggests that the intracellular enzymes which act upon nucleo-proteins are inhibited less by serum than are the enzymes which attack the simpler proteins of the cells, a view entirely in harmony with the results of chemical studies. Heating the serum to 80° for thirty minutes (removing the coagulum by filtration) greatly reduced its power to inhibit autolytic changes in the cell structure, although the changes in this menstruum were always somewhat less rapid than the changes that took place in similar tissues kept in salt solution.

From the above observations it would seem that the thermostable element of serum which inhibits autolysis of liver tissue is distinct from the thermolabile anti-leucoprotease described by Opie. However, there seems to be a discrepancy in the results obtained when the autolytic changes are measured by physical or chemical means, and when they are measured by the histological changes which take place in the autolyzing cells.

#### GENERAL CONSIDERATIONS.

We believe that these experiments have, above all, indicated the value of the results obtained by physico-chemical methods in the study of autolytic processes. Not only do these methods permit of a much greater number of determinations being made in less time with less material, and with less labor and expense, but they are also of far more value in giving an idea of the amount of change that is taking place in the autolyzing tissues, than are the chemical methods in common use. Autolysis comprises the disintegration of the cell components, and involves a great number of chemical substances, some of which are coagulable proteins and many of which are not. The customary measure of autolysis is the change in the amount of nitrogen contained in forms coagulable and not coagulable by heat—a measure which is very misleading and of com-



paratively little value for many reasons. In the first place it takes into account only the autolysis of the nitrogenous constituents of the cell which are either insoluble or heat coagulable. Secondly, it measures only a single step of the many changes that take place in these particular cellular elements—namely, their loss of coagulability; this is certainly a very inadequate measure of tissue disintegration, for it gives the same result whether a coagulable or insoluble protein is merely converted into soluble proteoses or whether the most extensive disintegration to the ultimate amino acids has been accomplished. Thirdly, the amount of connective tissue in the organ under investigation may modify greatly the amount of coagulable nitrogen, since during the heating and extraction the collagen will be converted into gelatin and will be estimated with the incoagulable nitrogen equally whether it has been attacked by the enzymes or not. Fourth, it takes no account of the extent of disintegration of the all-important nucleo-proteins, for after the first step of this disintegration is accomplished the products are soluble, and so all the other important later changes in the nucleic acid and the purines are not shown by the nitrogen determinations.

On the other hand the determination of the freezing point gives us an absolute, delicate and reliable measure of disintegration of the tissue, since every step of this disintegration results in the presence of more ions and molecules, no matter whether it is the proteins, the carbohydrates or the fats of the cell that breaks down. A freezing point curve is, therefore, a correct picture of the change that is taking place in the autolyzing tissue, which a non-coagulable nitrogen curve cannot well be. If we supplement the freezing point curve with a conductivity curve we secure information of qualitative changes that is of great value, for the conductivity curve indicates the increase of free ions, which we know are largely supplied by certain products of autolysis, while the difference between the two curves gives us a measure of the new non-electrolytes that have made their appearance as a result of disintegration of other non-electrolytes. The information obtained by these two methods of analysis is, therefore, not only more easily obtained but also much more instructive, and a much better indication of the actual amount of autolysis, than is the mere estimation of nitrogen in coagulable and non-coagulable form, even when in

addition we make determinations of nitrogen in proteose peptone forms.

Inhibition of autolysis by normal serum concerns only the alytic disintegration of certain of the cell constituents, particularly the coagulable proteins, and not all the enzymes which have to do with hydrolysis of cell constituents are thus inhibited. As the inhibiting effect of serum upon autolysis of the fixed tissue is destroyed readily by heat, it is presumably different from the therabile constituent of serum which inhibits autolysis and heterolysis by leucocytic enzymes.

# A METHOD OF TREATING AND PRESERVING LARGE QUANTITIES OF URINE FOR INORGANIC ANALYSIS.

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(Received for publication, May 3, 1910.)

A problem frequently to be faced in quantitative analysis is the ashing of large quantities of urine, either for the determination of metallic elements normally present in small amount or more especially for the determination of foreign metallic elements.

The method which would naturally suggest itself is the one commonly employed commercially in the analysis of water. This consists in allowing the water to flow from the containing vessel by gravity into a platinum dish, with the exit tube projecting just under the surface of the water in the dish. The flow of water from the vessel is then regulated to equal the rate of evaporation from the dish. It is at once evident that a method of this nature would not adapt itself to the ashing of a large quantity of urine.

When urine is evaporated, in the air, on the steam bath or hot plate a resinous sticky mass results which will not admit of complete drying and which, at about 100° C., evolves ammonia. Urine in this condition is not suitable for quantitative study.

To obviate the difficulties mentioned the method devised by A. Newman<sup>1</sup> has been widely used. This, in principle, consists in adding to the urine one-tenth its volume of concentrated nitric acid and allowing this mixture to drop into boiling concentrated nitric acid. The oxidation is completed by treatment with a mixture of equal quantities of sulphuric and nitric acid. While this method will completely oxidize the urine (in some cases repeated treatment with this mixed acid is necessary), it requires the constant

<sup>1</sup>*Arch. f. Physiol.*, 1902, p. 362.

attention of the operator and is rather tedious when a number of liters have to be ashed.

Sometime ago in the course of my work in connection with one of the patients in the medical wards, it became necessary to examine the urine in a case of metallic poisoning. A few preliminary tests proved the presence of a minute trace of copper. Subsequent work showed copper to be present in the amount of about 0.032 mg. per liter, calculated as metallic copper. This required the ashing of a large quantity of urine and the method which was devised at that time has turned out to be very convenient.

It was found that if sulphuric acid be added to urine and if evaporation then be allowed to take place, the residue dries hard, without spattering, and can be removed from the dish (sometimes in a solid cake) and can be pulverized.

Some care must be exercised as to the amount of acid added. If too much be used the urine yields on evaporation a tarry oil. If too little, it does not completely dry. Five cubic centimeters of sulphuric acid (1.98 sp. g.) per liter, should be used for urine with specific gravity ranging up to 1020. For pathological urines containing much organic matter (e.g., sugar) a larger amount of acid must be added. If the residue then does not dry a little sulphuric acid can be stirred into the mass and the heating continued.

The solid residue obtained in this manner resembles dried food or feces, or, if the oxidation be carried further, it may look like animal charcoal. It retains the odor peculiar to urine and dissolves in water to yield a solution which has the original color of the urine. The weight of the dry material ranges from 10-30 grams per liter depending upon the specific gravity of the urine.

This method allows one to place the urine on the water bath on leaving the laboratory in the evening and to find it ready for analysis on return in the morning.

While this method was originally intended to be used only in the determination of foreign metallic elements that may accidentally occur in the urine in small amount, further study has shown it to have a more important bearing. Sometimes, in the investigation of mineral metabolism, it is desirable to preserve the urine, together with the food and feces, for future study; indeed, it may often be desirable to delay urine analyses for some time. The urine on standing, though it contain thymol or chloroform, will finally

evolve ammonia and precipitate the phosphates which carry down other constituents and form a gelatinous mass which is hard to sample accurately. If the urine be first treated by the method described above, it can be sampled accurately, as experiments have shown, and since it is sampled by weight, the sampling is more accurate than is possible by volume.

By this procedure of analysis one should first note the daily quantity and take the specific gravity of the urine; the amount evaporated need not be measured, though it is convenient to use from one-half to one liter of the mixed twenty-four hours urine. After evaporation the material must be ground, sampled and weighed out for analysis. The ashing can be carried out with the proper flux in a crucible or in a Kjeldahl flask. The results of the analysis are expressed in per cent and since the specific gravity of the urine is known, one can readily compute the mineral contents in parts per liter or in the total amount of urine passed in the twenty-four hours.

To sum up: This method will be found to be of especial value in the determination of foreign inorganic elements occurring in the urine, and can be used in the determination of all bases except ammonia. It will also be found to be of value in the determination of the bases in urine, in the presence of albumen, since in albuminuria ashing of the urine is necessary in order that these determinations may be made. In addition, it will be of value, as mentioned above, when very accurate determinations of the inorganic basic constituents are desired and when the analysis of the urine is deferred.



## PHOSPHORUS IN BEEF ANIMALS.<sup>1</sup> PART II.

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(Received for publication, April 22, 1910.)

### *Distribution of Phosphorus in Tissues and Organs.*

The fresh samples were analyzed by the methods previously described.<sup>2</sup> Moisture and fat, ash and total phosphorus were determined on separate samples in triplicate. The average results are reported.

Eight animals are considered in the following tables. Animals 592, 595, 597, and 594 were young and in various conditions as indicated in Table II, previous paper. The next four, 18, 121, 48, and 43 were mature; also varying in condition.

### *Method of Obtaining Samples.*

The samples of blood and the organs were obtained at the time of slaughtering. Forty-eight hours later, when the right half of the carcass was cut into wholesale cuts, the lean, fat and bone samples were taken for analysis. In each case the entire cut was ground, as previously described, before the sample was sent to the laboratory.

### *Samples Analyzed.*

The following organs, tissues, etc., were analyzed. In many cases, owing to lack of time, it was necessary to combine several parts; these are enclosed in brackets following the designation under which

<sup>1</sup> This is a continuation of the previous paper, *This Journal*, vii, p. 481, 1910.

<sup>2</sup> *Ibid.*

they appear in the tables of results. Analyses of bone samples are not included in this paper.

Blood: Liver: Digestive and excretory system [tongue (total, less bones), gullet, stomach (clean, less fat), intestines (clean, less fat), spleen, pancreas, thymus (neck), thymus (heart), gall bladder and contents, kidneys, bladder (less contents), penis (or uterus and vagina), diaphragm (skirt)]: Circulatory system [heart, pericardium and fat, arteries]: Respiratory system [trachea, lungs and fat]: Nervous system brain, spinal cord]: Hair and hide: Offal fat [omentum or caul, fat from intestines, fat from stomach]: Kidney fat: Lean and fat of head and tail: Lean and fat of shin and shank: Lean of round: Fat of round: Lean and fat of rump: Lean of loin: Fat of loin: Lean and fat of chuck and neck: Lean and fat of flank and plate: Lean of rib: Fat of rib: Udder.

It was not possible to adhere to the above outline with every animal, but any change made will appear in the following tables of analyses.

TABLE I.  
*Composition of Cuts and Organs of Steer No. 592.*

LAB. NO.	SAMPLES	PER CENT MOISTURE	PER CENT ASH	PER CENT PHOSPHORUS	PER CENT FAT
9151	Blood .....	83.68	0.800	0.019	none
9158	Hair and hide .....	61.06	1.178	0.039	0.49
9154	Circulatory system .....	77.41	0.923	0.137	5.49
9155	Respiratory system.....	79.11	1.162	0.159	5.56
9156	Nervous system .....	76.21	1.407	0.323	9.78
9157	Digestive and excretory system. ....	82.89	0.863	0.183	3.61
9152	Liver.....	71.34	1.540	0.333	3.04
9179	Kidney fat.....	81.42	1.213	0.067	4.59
9178	Offal fat.....	81.60	1.055	0.109	5.03
9169	Head and tail <sup>1</sup> .....	71.89	1.071	0.160	8.69
9170	Shin and shank.....	76.37	0.979	0.164	1.21
9175	Chuck and neck .....	77.84	1.040	0.170	1.52
9173	Flank .....	75.35	0.937	0.130	0.90
9172	Rump .....	77.36	1.089	0.173	3.04
9171	Round .....	77.25	1.070	0.184	2.01
9174	Loin .....	77.16	1.076	0.179	1.62
9176	Plate .....	75.10	0.996	0.146	3.06
9177	Rib .....	77.01	1.084	0.168	2.09
9168	Composite of leans and fats .....	76.37	1.045	0.174	1.87

<sup>1</sup>In all carcass cuts lean and fat were combined for analysis, if not otherwise stated.



TABLE II.

*Composition of Cuts and Organs of Steer No. 595.*

I.A.B. NO.	SAMPLES	PER CENT	PER CENT	PER CENT	PER CENT
		MOISTURE	ASH	PHOSPHORUS	FAT
9251	Blood .....	80.63	0.747	0.029	none
9258	Hair and hide .....	64.09	1.422	0.061	1.47
9254	Circulatory system .....	63.68	0.744	0.145	21.65
9255	Respiratory system .....	77.99	1.013	0.179	4.05
9256	Nervous system .....	67.21	1.492	0.361	18.60
9257	Digestive and excretory system .....	75.34	0.784	0.148	10.92
9252	Liver .....	70.85	1.473	0.319	3.39
9279	Kidney fat .....	26.88	0.334	0.050	69.66
9278	Offal fat .....	31.36	0.319	0.050	62.23
9269	Head and tail lean .....	72.88	0.961	0.182	7.33
9281	Head and tail fat .....	49.89	0.587	0.071	32.28
9270	Shin and shank lean .....	75.08	0.997	0.187	3.00
9282	Shin and shank fat .....	51.45	0.629	0.065	27.62
9271	Round lean .....	75.86	1.044	0.203	2.41
9283	Round fat .....	40.07	0.495	0.062	45.19
9272	Rump lean .....	73.29	1.050	0.205	5.60
9284	Rump fat .....	26.40	0.419	0.069	62.39
9273	Flank and plate lean .....	70.63	0.983	0.180	8.34
9285	Flank and plate fat .....	36.94	0.516	0.058	46.84
9274	Loin lean .....	73.21	1.003	0.199	5.48
9286	Loin fat .....	27.65	0.419	0.064	62.78
9275	Chuck and neck lean .....	74.73	0.998	0.187	4.21
9287	Chuck and neck fat .....	41.67	0.529	0.080	46.04
9277	Rib lean .....	72.82	0.996	0.191	5.67
9289	Rib fat .....	26.96	0.554	0.082	58.13
9268	Lean composite .....	73.52	0.997	0.196	4.91
9280	Fat composite .....	36.79	0.492	0.069	48.54

TABLE III.

*Composition of Cuts and Organs of Steer No. 597.*

LAB. NO.	SAMPLES	PER CENT MOISTURE	PER CENT ASH	PER CENT PHOSPHORUS	PER CENT FAT
8951	Blood .....	80.60	0.339	0.025	none
8958	Hair and hide.....	61.60	0.646	0.051	2.29
8954	Circulatory system.....	51.37	0.764	0.125	37.15
8955	Respiratory system .....	74.75	0.956	0.162	6.16
8956	Nervous system .....	71.26	1.726	0.417	13.20
8957	Digestive and excretory system.....	71.74	0.672	0.122	15.19
8952	Liver.....	70.30	1.376	0.332	2.70
8980	Kidney fat .....	7.50	0.115	0.014	90.22
8979	Offal fat .....	17.97	0.462	0.032	77.88
8970	Head and tail lean .....	70.97	0.859	0.164	8.98
8982	Head and tail fat .....	35.69	0.435	0.061	49.97
8971	Shin and shank lean.....	73.48	0.881	0.170	4.07
8983	Shin and shank fat.....	40.19	0.326	0.046	44.31
8972	Round lean .....	74.32	0.956	0.193	3.69
8984	Round fat.....	26.64	0.285	0.052	63.61
8973	Rump lean .....	70.77	0.919	0.185	8.18
8985	Rump fat.....	18.18	0.223	0.040	74.18
8974	Flank lean.....	67.08	0.907	0.176	11.35
8986	Flank fat.....	22.24	0.215	0.031	68.20
8977	Plate lean .....	63.90	0.762	0.148	16.18
8989	Plate fat .....	24.90	0.253	0.038	66.17
8975	Loin lean.....	71.64	0.945	0.183	5.94
8987	Loin fat .....	18.96	0.250	0.044	73.76
8976	Chuck and neck lean .....	73.07	0.866	0.171	5.69
8988	Chuck and neck fat .....	29.46	0.315	0.055	58.72
8978	Rib lean.....	69.01	0.862	0.164	10.26
8990	Rib fat .....	21.86	0.286	0.051	70.19
8969	Lean composite.....	71.29	0.928	0.178	8.54
8981	Fat composite .....	25.49	0.292	0.048	62.82

TABLE IV.

*Composition of Cuts and Organs of Steer No. 594.*

LAB. NO.	SAMPLES	PER CENT MOISTURE	PER CENT ASH	PER CENT PHOSPHORUS	PER CENT FAT
8232	Blood .....	79.34	0.318	0.055	none
8235	Hair and hide .....	64.38	0.965	0.072	3.62
8240	Circulatory, respiratory and nervous systems .....	65.74	0.863	0.181	18.94
8221	Digestive and excretory systems.....	71.70	0.932	0.193	12.27
8233	Liver .....	68.82	1.340	0.347	5.27
8244	Kidney fat .....	5.48	0.072	0.020	93.16
8245	Offal fat .....	10.96	0.170	0.034	85.87
8288	Shin, shank, head, tail, lean .....	73.64	0.916	0.182	4.94
8289	Shin, shank, head, tail, fat .....	41.91	0.445	0.077	44.84
8290	Round lean .....	72.64	1.024	0.207	5.34
8297	Round fat .....	20.75	0.243	0.043	71.01
8291	Rump lean .....	71.22	1.023	0.212	7.23
8298	Rump fat .....	13.52	0.181	0.036	82.36
8292	Loin lean .....	70.22	0.986	0.191	8.30
8299	Loin fat .....	13.41	0.168	0.031	82.47
8293	Flank lean .....	65.64	0.905	0.176	13.71
82100	Flank fat .....	19.57	0.151	0.025	72.85
8294	Plate lean .....	65.09	0.861	0.170	15.47
82101	Plate fat .....	22.99	0.268	0.044	69.75
8295	Rib lean .....	68.90	0.856	0.171	10.53
82102	Rib fat .....	14.27	0.202	0.035	80.82
8296	Chuck and neck lean .....	72.95	0.916	0.178	6.81
82103	Chuck and neck fat .....	23.29	0.269	0.046	70.25
8242	Lean composite .....	71.31	0.985	0.193	6.88
8243	Fat composite .....	20.05	0.231	0.044	72.90

TABLE V.  
*Composition of Cuts and Organs of Steer No. 18.*

LAB. NO.	SAMPLES	PER CENT MOISTURE	PER CENT ASH	PER CENT PHOSPHORUS	PER CENT FAT
71156	Blood .....	81.29	0.688	0.022	none
71157	Hair and hide .....	65.36	0.868	0.068	2.88
71167	Circulatory system .....	56.50	0.751	0.123	30.07
71168	Respiratory system .....	74.71	0.959	0.151	6.46
71161	Nervous system .....	68.83	1.757	0.422	17.62
71166	Digestive and excretory system .....	66.51	1.634	0.129	18.13
71189	Kidneys .....	75.05	1.085	0.211	8.74
71162	Liver.....	68.99	1.253	0.311	3.47
71190	Kidney fat .....	10.04	0.120	0.021	86.96
71165	Offal fat.....	16.62	0.172	0.027	79.72
71180	Shin, shank, head, and tail.....	67.43	0.771	0.145	11.99
71181	Round and rump.....	66.50	0.883	0.172	13.26
71182	Loin .....	59.90	0.788	0.157	21.72
71183	Flank and plate.....	52.51	0.649	0.124	30.10
71184	Rib .....	62.79	0.821	0.161	18.04
71185	Chuck and neck .....	66.48	0.818	0.154	14.07

TABLE VI.

*Composition of Cuts and Organs of Steer No. 121.*

LAB. NO.	SAMPLES	PER CENT MOISTURE	PER CENT ASH	PER CENT PHOSPHORUS	PER CENT FAT
71250	Blood .....	77.97	0.248	0.028	none
71262	Hair and hide .....	54.42	0.758	0.056	4.97
71254	Heart lean .....	77.38	1.000	0.211	4.39
71255	Circulatory system less heart.....	27.42	0.205	0.039	65.27
71256	Respiratory system .....	63.96	0.808	0.170	19.69
71257	Nervous system .....	68.36	1.562	0.395	19.50
71258	Digestive and excretory system less stomach, tongue, liver and kidneys.	55.54	0.633	0.126	31.73
71274	Stomach.....	78.26	0.971	0.200	8.21
71253	Tongue edible .....	60.40	0.642	0.132	25.27
71251	Liver .....	68.36	1.312	0.353	4.72
71260	Kidneys .....	76.58	0.950	0.229	5.56
71261	Kidney fat .....	4.48	0.070	0.012	94.67
71259	Offal fat.....	9.52	0.129	0.022	88.02
71265	Shin, shank, head, and tail.....	62.30	0.733	0.142	18.18
71273	Chuck and neck .....	59.14	0.702	0.142	24.43
71266	Round and rump lean.....	69.96	0.957	0.191	8.18
71267	Round and rump fat .....	14.41	0.153	0.030	80.61
71268	Loin lean.....	67.22	0.943	0.185	11.40
71269	Loin fat .....	9.08	0.133	0.024	87.84
71270	Flank and plate .....	40.07	0.500	0.095	47.70
71271	Rib lean.....	60.98	0.777	0.153	20.87
71272	Rib fat.....	10.68	0.135	0.027	86.04

TABLE VII.  
*Composition of Cuts and Organs of Steer No. 48.*

LAB. NO.	SAMPLES	PER CENT MOISTURE	PER CENT ASH	PER CENT PHOSPHORUS	PER CENT FAT
91101	Blood .....	79.41	0.775	0.021	none
91112	Hair and hide .....	59.24	0.197	0.048	8.60
91105	Heart marketable.....	65.83	0.835	0.158	19.45
91104	Circulatory system less heart.....	10.07	0.145	0.026	88.45
91106	Respiratory system .....	49.42	0.669	0.117	37.39
91107	Nervous system .....	69.63	1.813	0.425	13.05
91108	Digestive and excretory system less tongue, stomach and kidney and liver.	64.16	0.697	0.128	22.41
91110	Stomach.....	74.75	1.073	0.227	11.94
91109	Tongue edible .....	56.02	0.743	0.126	29.50
91102	Liver.....	69.73	1.392	0.307	4.26
91111	Kidneys .....	70.75	1.154	0.199	12.16
91134	Kidney fat .....	3.76	0.126	0.016	94.71
91135	Offal fat.....	6.22	0.093	0.012	92.09
91120	Shin, shank, head and tail.....	57.84	0.829	0.142	23.47
91121	Chuck and neck .....	49.67	0.693	0.123	34.95
91122	Flank and plate.....	30.15	0.421	0.064	61.11
91123	Rump .....	36.91	0.509	0.093	52.67
91124	Round lean.....	64.43	1.015	0.192	13.42
91125	Round fat .....	27.75	0.251	0.027	67.35
91126	Loin lean.....	61.78	0.956	0.174	18.26
91127	Loin fat.....	12.60	0.146	0.023	84.76
91128	Rib lean.....	56.86	0.827	0.150	24.74
91129	Rib fat .....	15.81	0.155	0.021	81.37

TABLE VIII.

*Composition of Cuts and Organs of Cow No. 43.*

LAB. NO.	SAMPLES	PER CENT MOISTURE	PER CENT ASH	PER CENT PHOSPHORUS	PER CENT FAT
91051	Blood.....	80.40	0.742	0.030	none
91057	Hair and hide .....	63.30	1.252	0.072	4.27
91054	Circulatory system.....	47.45	0.505	0.103	46.74
91055	Respiratory system.....	71.86	0.902	0.164	12.84
91056	Nervous system.....	72.72	1.441	0.354	13.48
91053	Digestive and excretory system.....	76.66	0.736	0.140	10.80
91052	Liver.....	72.17	1.341	0.339	2.21
91089	Kidney fat.....	6.14	0.095	0.016	94.29
91058	Offal fat.....	11.63	0.074	0.020	88.20
91068	Head and tail lean.....	68.81	0.889	0.169	13.41
91078	Head and tail fat.....	27.64	0.345	0.044	68.24
91069	Shin and shank lean.....	72.44	1.067	0.196	5.65
91079	Shin and shank fat.....	53.49	0.618	0.061	35.27
91070	Round lean.....	73.20	1.026	0.195	6.92
91080	Round fat.....	30.25	0.406	0.059	65.23
91071	Rump lean.....	67.67	0.955	0.185	12.71
91081	Rump fat .....	24.02	0.225	0.035	79.34
91072	Loin lean.....	71.11	0.959	0.186	12.27
91082	Loin fat.....	16.40	0.189	0.029	82.97
91073	Chuck and neck lean.....	67.27	0.968	0.184	12.33
91083	Chuck and neck fat.....	19.96	0.247	0.039	78.00
91074	Flank lean.....	67.37	1.016	0.188	13.52
91084	Flank fat.....	26.04	0.289	0.029	70.87
91075	Plate lean.....	63.27	0.888	0.165	18.80
91085	Plate fat.....	23.00	0.250	0.041	79.23
91076	Rib lean.....	65.45	0.907	0.173	17.87
91086	Rib fat.....	22.86	0.317	0.044	78.45
91077	Composite of leans.....	69.86	0.966	0.186	12.71
91087	Composite of fats.....	19.50	0.247	0.033	78.75
91088	Udder.....	69.37	0.805	0.138	17.19

TABLE IX.

Summary—Phosphorus Calculated to Water and Fat Free Conditions, Per Cent

NO. OF ANIMAL	592		595		597	
Condition	Fresh	Moisture and Fat Free	Fresh	Moisture and Fat Free	Fresh	Moisture and Fat Free
Blood .....	0.019	0.116	0.029	0.149	0.025	0.116
Hair and hide .....	0.039	0.101	0.061	0.177	0.051	0.101
Circulatory system .....	0.137	0.801	0.145	0.988	0.125	1.000
Respiratory system .....	0.159	1.037	0.179	0.996	0.162	0.996
Nervous system .....	0.323	2.305	0.361	2.544	0.417	2.600
Digestive and excretory system.	0.183	1.356	0.148	1.077	0.122	0.996
Liver .....	0.333	1.299	0.319	1.238	0.332	1.238
Kidney fat.....	0.067	0.479	0.050	1.445	0.014	0.600
Offal fat .....	0.109	0.815	0.050	0.780	0.032	0.780
Shin, shank, head, and tail.....	0.163	0.762	0.175	0.822	0.145	0.780
Round .....	0.184	0.887	0.192	0.905	0.171	0.887
Rump .....	0.173	0.882	0.178	0.927	0.141	0.887
Loin .....	0.179	0.844	0.185	0.917	0.145	0.780
Flank and plate .....	0.142	0.637	0.162	0.796	0.114	0.600
Rib .....	0.168	0.804	0.189	0.883	0.148	0.780
Chuck and neck .....	0.170	0.824	0.181	0.881	0.153	0.780
Composite of leans and fats.....	0.174	0.799	0.185	0.877	0.151	0.887





## SUMMARY.

In Table IX the phosphorus content for all samples has been calculated to a moisture and fat-free condition. The results for the young animals, 592, 595, 597 and 594, are quite uniform among themselves, but this is not so striking for the other four mature animals.

The largest amount of phosphorus was found in the circulatory systems and the nervous systems. Two of the mature animals, a cow (43) and a steer (48), showed abnormally high results in the circulatory systems. Two animals, 18—which was 3.5 years old and very thin—and 121—which was also 3.5 years old, but fairly fat—contained more phosphorus in the nervous systems than the other animals. The Jersey cow, which was the oldest animal examined, showed the highest average amount of phosphorus.

So far as the phosphorus in the flesh of the cuts is concerned, it is impossible to draw any final conclusion with the amount of data available. Steer 594, young, fat, and in a very thrifty condition, contained more phosphorus—moisture and fat-free basis—than steers 18, 121 and 48, three mature steers. It was also superior in this respect to 592, 595 and 597, which were young, but not in so thrifty a condition. The mature cow, however, showed in the flesh cuts a higher phosphorus content than any of the other animals discussed. This can hardly be attributed to condition, because 121 was fully as fat, and 48 much fatter. Whether it was due to age is a little doubtful, as she was only two years older than 48. We are more inclined to think that the breed or the high phosphorus diet (bran, etc.) previous to the fattening period may have been influential.

The wholesale cuts of the seven steers show an increasing amount of phosphorus, compared on the moisture and fat-free basis, in the following order: flank and plate; shin, shank, head and tail; rib, chuck and neck; loin; round; rump. In other words, those cuts thin in character and which have the largest amount of connective tissue contain the smallest amount of phosphorus.

It is remarkable that the very thin steer 592, while comparatively low in phosphorus, showed a higher percentage of ash in every cut than any of the other animals. Steer 595, also thin, contained some-

what less, but still higher than the other remaining animals. Steer 121, well fed and in excellent condition, gave comparatively low results in ash. It is to be noted that there seems to be no relation between the phosphorus and the ash. An explanation of the fact cannot be attempted until the analyses of the various samples of ash are completed.



## NOTE ON CHEMICAL TESTS FOR BLOOD.

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(Received for publication, May 10, 1910.)

Color reactions, produced by the oxidizing property of blood on guaiacum, aloin, benzidin, leuco-base of malachite green and phenolphthalin<sup>1</sup> have been used for a long time to detect small quantities of blood in the gastric contents and are of diagnostic importance in determining the presence of ulcer or carcinoma of the stomach. Most observers recognize the fact that while other substances will give positive reactions for blood, a negative result is a proof of its absence.

In using the Ewald-Boas test-meal of 35 grams of bread and 400 cc. of weak tea or water, we have found that stomach contents known to contain blood gave no reaction, even after adding it. Further investigation showed that this occurred only in those cases in which tea had been given with the test meal. This suggested tannic acid as the inhibitor and it was demonstrated *that solutions of tannic or gallic acid totally prevented dilute blood reactions; tea and coffee extracts acted in practically the same way.* Thus far our experiments show that these inhibitors also affect all peroxide catalysors, including iron and copper.

The amount of inhibitor and the quantity of blood seem to be definitely related, as five volumes of 1-1000 tannic or gallic acid inhibit approximately one volume of 1-1000 blood.

### CONCLUSION.

In using the Ewald-Boas test meal, water, and not tea, should be given, as an accurate qualitative and quantitative determination of blood cannot be made when either tannic or gallic acid is present.

<sup>1</sup> J. H. Kastle, Chemical Test for Blood, Bull. no. 51, Hygienic Laboratory, Public Health and Marine Hospital Service of U. S.



# THE FORMATION IN THE ANIMAL BODY OF $l$ - $\beta$ -OXY-BUTYRIC ACID BY THE REDUCTION OF ACETO-ACETIC ACID.

## A CONTRIBUTION TO THE STUDY OF ACIDOSIS.

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(Received for publication, June 23, 1910.)

It is well known that  $\beta$ -oxybutyric acid and substances capable of giving rise to this acid may undergo oxidation in the liver with formation of aceto-acetic acid and acetone. This has been most convincingly demonstrated by the striking experiments of Embden and his co-workers upon the perfusion of surviving livers with blood containing  $\beta$ -oxybutyric acid, etc. The reaction involved in this oxidation of  $\beta$ -oxybutyric acid has been shown by Wakeman and the writer to be due to an oxidizing enzyme whose action was facilitated by the presence of free oxygen and of oxyhæmoglobin or blood.<sup>1</sup> One of the main objects of the following paper is to point out the reversible nature of this reaction. If aceto-acetic acid in the form of its sodium salt be injected intravenously into cats or dogs, a reduction occurs of a considerable part of the salt with formation of lævo-rotatory  $\beta$ -oxybutyric acid which is excreted in the urine. Thus the injection of 12 grams of aceto-acetic acid was followed by the elimination of 1 to 2 grams of  $\beta$ -oxybutyric acid. This fact was recorded in a short paper<sup>2</sup> sent on March 16 to the *Journal of the American Medical Association*, which, however, notwithstanding a promise of prompt publication, did not appear until April 29. In the meantime a paper appeared by Blum<sup>3</sup> (March

<sup>1</sup> This *Journal*, vi, p. 373, 1909.

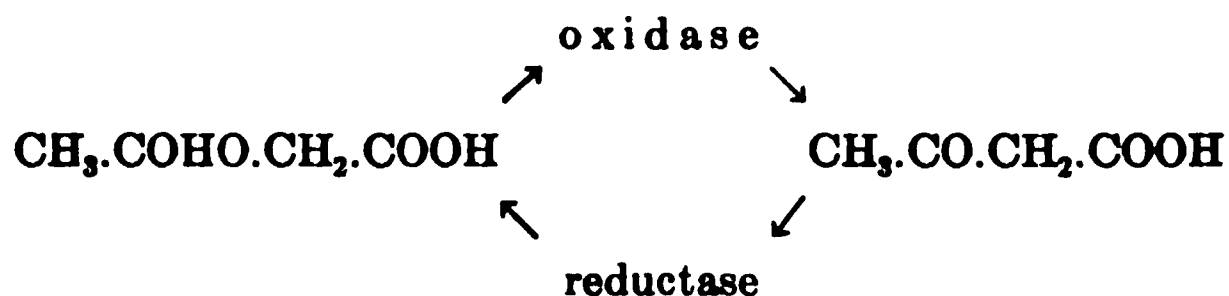
<sup>2</sup> *Journ. Amer. Med. Assoc.*, liv, No. 18, p. 1441, 1910.

<sup>3</sup> *Munch. med. Woch.*, lvii, p. 683, 1910. Reference is made by Blum to some experiments of Maase along similar lines, the publication of which, so far has not been accessible to me.

29) in which the same fact was established by slightly different methods. Blum's paper contains in addition many other results which have an important bearing upon the problem of fatty acid catabolism.

Further experiments made by Wakeman and the writer have shown that ground liver tissue when digested outside the body with sodium aceto-acetate also forms  $l$ - $\beta$ -oxybutyrate, so that it may reasonably be concluded that the change is due to enzyme action (cf. following paper).

*It will thus be seen that the liver possesses a mechanism, dependent upon the antagonistic action of two ferments, by which the mutual interconversion of  $\beta$ -oxybutyric acid and aceto-acetic acid may be effected. The one ferment action is an oxidation dependent upon the presence of free oxygen or oxyhaemoglobin while the other ferment action must be of the nature of a reduction. It appears probable that under some circumstances the reducing reaction is quantitatively predominant.*



When the pathological conditions associated with diabetes resulting in the excretion of  $l$ - $\beta$ -oxybutyric acid in the urine are considered, the possibility at once presents itself that the  $\beta$ -oxybutyric acid in large measure is formed *not* from the oxidation of butyric acid as is commonly supposed but from the reduction of aceto-acetic acid. There appears to be evidence in support of this view. In the first place  $\beta$ -oxybutyric acid is always associated in the urine with aceto-acetic acid, so that excess of the latter substance is undoubtedly present in the tissues, which, as has been demonstrated, possess the power of reducing the ketonic acid to  $l$ - $\beta$ -oxybutyric acid.

In the second place, Blum, in the paper referred to, produces strong evidence pointing to the belief that aceto-acetic acid and not  $\beta$ -oxybutyric acid is the primary product of the oxidation in the body of butyric acid. In this connection it may be recalled that the writer when investigating the oxidation of salts of butyric



acid with hydrogen peroxide was unable to detect  $\beta$ -oxybutyric acid although aceto-acetic acid and acetone were formed in abundance.

In the third place experiments with excised livers and also with intact animals appear to show that the change involved in reducing aceto-acetic acid to  $\beta$ -oxybutyric acid is more readily effected than the reverse change, namely, the oxidation of  $\beta$ -oxybutyric acid to aceto-acetic acid. Thus the writer has administered 10 grams of oxybutyric acid in the form of its sodium salt to a rabbit weighing 1.7 kilos. The urine secreted during the succeeding twenty-four hours contained no aceto-acetic acid and showed only a slight increase in acetone, although unchanged  $\beta$ -oxybutyric acid was present.<sup>1</sup>

The administration of a corresponding quantity of sodium acetoacetate results in the excretion of considerable amounts of sodium *l*- $\beta$ -oxybutyrate.

In this connection it may be mentioned that 100 grams of fresh dog liver when digested with excess of either sodium  $\beta$ -oxybutyrate or sodium acetoacetate under the conditions adopted by Wakeman and the writer in their previous experiments, will oxidize on an average about 35 milligrams of  $\beta$ -oxybutyric acid but will reduce on an average about 150 milligrams of aceto-acetic acid.

Blum has also published similar experiments on the fate of sodium  $\beta$ -oxybutyrate.

*On the whole, therefore, it appears not unlikely that the underlying cause associated with  $\beta$ -oxybutyric acid acidosis is to be sought in the defective catabolism of aceto-acetic acid rather than that of the  $\beta$ -oxybutyric acid, which results from the reduction in the liver of the latter substance.*

The question arises as to whether the mutual interconversion of *l*- $\beta$ -oxybutyric acid and aceto-acetic acid brought about by reducing and oxidizing ferment action is due to the action of one or of two or more enzymes. It might be considered that, since the reaction in question is in a sense reversible, the change could be brought about by one ferment acting in opposite directions in the

<sup>1</sup> McKenzie (*Trans. Chem. Soc.*, lxxxi, p. 1499) found both aceto-acetic acid and acetone in the urine of dogs that had received salts of inactive  $\beta$ -oxybutyric acid administered subcutaneously.

same way as lipase, for example, both hydrolyzes fats and synthesizes them from their components. The writer regards this as unlikely for the following reasons: firstly because it appears possible to partially separate the two enzyme actions; thus an aqueous cell-free extract of liver tissue has the power to oxidize  $\beta$ -oxybutyric acid to aceto-acetic acid but apparently no action on the latter substance. In the second place it is not improbable that the mechanism of a reducing reaction such as is concerned in the reduction of aceto-acetic acid is very different from the reversed oxidation of  $\beta$ -oxybutyric acid in which, apparently, free oxygen is necessary. Until further evidence is available it will probably be advisable to regard the two ferment reactions as separate and distinct although differing as regards products only in the direction of the change.

It would be highly interesting if the mechanism of the reduction in the liver of aceto-acetic acid to *l*- $\beta$ -oxybutyric acid could be elucidated. The reaction is of interest as furnishing a biological example of an asymmetric synthesis closely allied to the reaction studied by Cohen and Whitely<sup>1</sup> and by McKenzie<sup>2</sup> and others in which only partial success was attained in achieving asymmetric synthesis without employing biochemical methods. In the ferment reaction under consideration the aceto-acetic acid contains no asymmetric carbon atom, but one is introduced in the process of reduction. The fact that the product of reduction of the inactive ketonic acid should itself be optically active is doubtless to be sought in the combination of the aceto-acetic acid with asymmetric substances—in all probability with the reducing enzyme itself.

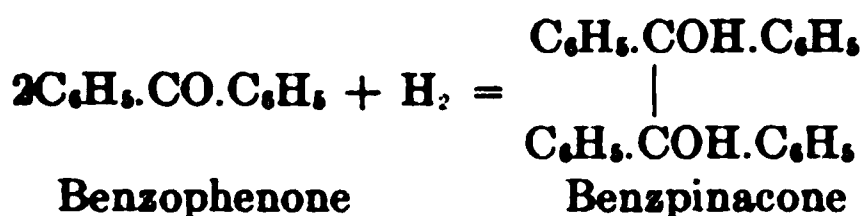
The reduction of aceto-acetic acid to  $\beta$ -oxybutyric acid involves the addition of two atoms of hydrogen to the former substance. A knowledge of the exact mode by which this hydrogen is furnished would be of the greatest interest. That elementary hydrogen is added to the aceto-acetic acid as in the reduction of aceto-acetic ester to  $\beta$ -oxybutyric ester by sodium amalgam is very unlikely.

There are at least two other possible methods of reduction which appear more likely to present biological analogies. Thus it might be found that some other easily oxidizable substance furnishes the

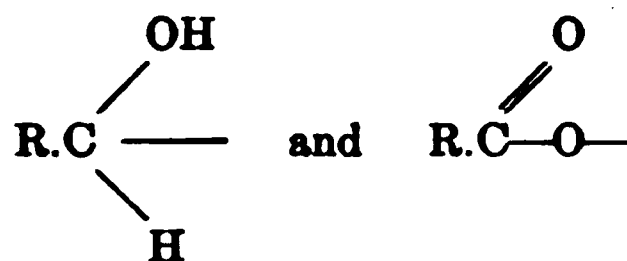
<sup>1</sup> *Trans. Chem. Soc.*, lxxix, p. 1305.

<sup>2</sup> *Ibid.*, lxxxvii, p. 1373 and later papers.

necessary hydrogen by itself undergoing oxidation. The beautiful work of Ciamician and Silber,<sup>1</sup> upon the influence of light upon chemical reactions has furnished a number of examples in which a ketonic compound has undergone reduction in alcoholic solution; a part of the alcohol being oxidized to acetaldehyde. Thus, for example, benzophenone may be reduced to benzopinacone under the influence of sunlight when dissolved in alcohol or in many other organic substances:



Another possible mode of formation of  $\beta$ -oxybutyric acid from aceto-acetic acid might be found in some change involving the simultaneous decomposition of two molecules of the ketonic acid, one of which undergoes oxidation whilst the other undergoes reduction. Although this particular type of change has not to my knowledge been observed with  $\beta$ -ketonic acids, there are innumerable examples of reactions in which substances containing the R.CO-group under the influence of alkali are converted into substances of the following type:

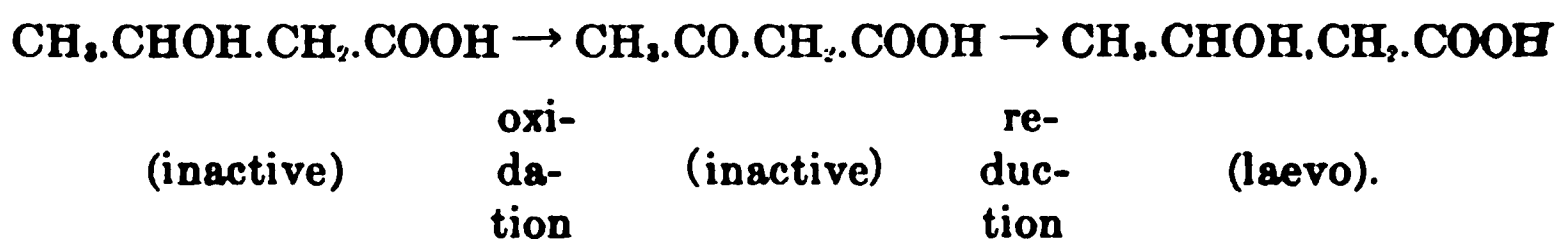


Perhaps the simplest example of such a simultaneous oxidation and reduction is the conversion of benzaldehyde into benzyl alcohol and benzoic acid under the influence of alkali.

The behavior of  $\beta$ -oxybutyric acid in the animal organism presents a stereo-chemical anomaly which may be briefly touched upon. In the case of the amino-acids comprising the products of hydrolysis of proteins it is the rule that the optically active form actually present in natural proteins undergoes decomposition in the animal body with greater ease than their optical isomerides. In other words the naturally occurring substance appears to be more available for the metabolic activities of living cells. Thus when inac-

<sup>1</sup> *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 1536.

tive tyrosine is fed to an animal, any tyrosine excreted in the urine is found to contain an excess of the dextro-component, *i.e.*, the *lævo* or natural component is preferentially attacked. With  $\beta$ -oxybutyric acid the relations are here reversed, for on administering inactive  $\beta$ -oxybutyric acid to an animal, any unchanged  $\beta$ -oxybutyric acid excreted in the urine is found to contain an excess of the *lævo* or natural component. This fact was first determined by McKenzie and has been frequently confirmed by the writer. At first sight, the conclusion might appear warranted that dextro- $\beta$ -oxybutyric acid was metabolized by the body at a faster rate than the natural or *lævo* isomeride. I believe this conclusion to be unwarranted for the following reason. It is known from the experiments already referred to that  $\beta$ -oxybutyric acid may be oxidized to aceto-acetic acid and also that this latter acid, which is of course optically inactive, may be again reduced to *lævo*- $\beta$ -oxybutyric acid. A consideration of these facts clearly indicates how conditions may arise by which the administration of inactive- $\beta$ -oxybutyric acid may give rise to an excretion of *l*- $\beta$ -oxybutyric acid in the urine as the result of the asymmetric reduction of aceto-acetic acid and not necessarily on account of a preferential metabolism of the dextro- $\beta$ -oxybutyric acid.



The observation of the reduction in the liver of aceto-acetic acid to *l*- $\beta$ -oxybutyric acid leads to the closely related question as to whether the *l*-phenyl- $\beta$ -oxypropionic acid found among the products of catabolism of phenyl-propionic acid does not originate at least in part from the reduction of benzoylacetic acid. There is some evidence available in support of this view and further experiments are already in progress.

#### EXPERIMENTAL.

##### *The Intravenous Administration of Sodium Aceto-acetate.*

These experiments were made by injecting carefully neutralized solutions of sodium aceto-acetate (prepared in the usual way by

saponification of the ester) into the femoral vein of cats and dogs. In most of the experiments ether was given during the insertion of the canula, in one experiment chloretone and morphine were used and in one case no anæsthetic was employed. No difference could be detected in the results. The urine passed during the injection and afterwards was collected by catheter and analyzed for  $\beta$ -oxybutyric acid. Acetone and unchanged aceto-acetic acid were invariably present in the strongly alkaline urine, which was concentrated on the water-bath to small bulk, acidified strongly with phosphoric acid and extracted in a continuous extractor with ether. In some cases the concentrated urine was mixed with gypsum and extracted by Black's method. The ethereal extract was dissolved in hot water, clarified with charcoal and then examined in the polarimeter. The amount of *l*- $\beta$ -oxybutyric acid was calculated from these readings and the aqueous solutions were subsequently examined chemically for  $\beta$ -oxybutyric acid. On oxidation with chromic acid according to Shaffer's method a large amount of acetone was readily obtained and identified by conversion into the para-nitrophenylhydrazone, melting point,  $149^{\circ}$ , while on distillation with sulphuric acid in the usual way, a considerable amount of crotonic acid was easily obtained. The crotonic acid was crystallized from water and melted at  $72-73^{\circ}$ .

**Analysis:**

0.1376 gram gave 0.2798 gm.  $\text{CO}_2$  and 0.0884 gm.  $\text{H}_2\text{O}$

	Calculated for $\text{C}_4\text{H}_6\text{O}_7$ :	Found:
C.....	55.8 per cent	55.5 per cent
H.....	7.0 per cent	7.1 per cent

The following are typical results:

(a). Cat weighing 4 kilos, received 12.0 grams of aceto-acetic acid as sodium salt (8 per cent solution), by femoral vein in the course of 6 hours. Urine, on analysis, gave rotation of ether extract equivalent to 1.07 grams of *l*- $\beta$ -oxybutyric acid.

(b). Puppy, weighing 3 kilos, received 10 grams of aceto-acetic acid as sodium salt. Animal died at end of 4 hours. Urine contained 0.67 gram of *l*- $\beta$ -oxybutyric acid.

(c). Large dog received 12 grams of aceto-acetic acid as sodium salt in course of 4 hours. Dog made perfect recovery. Urine contained 1.51 grams of  $\beta$ -oxybutyric acid.

(*d*). Dog, 12 kilos, received 12 grams of aceto-acetic acid as sodium salt in course of 6 hours. Urine contained 1.98 grams of  $\beta$ -oxybutyric acid.

(*e*). Similar experiment to (*c*). Urine contained 1.15 grams of  $\beta$ -oxybutyric acid.

*Experiments with Inactive- $\beta$ -oxybutyric Acid.*

(*f*). 10.0 grams of synthetic  $\beta$ -oxybutyric acid was exactly neutralized with caustic soda and given by mouth in 7 per cent solution to a rabbit weighing 1700 grams. The urine showed only a trifling increase in acetone. The urine was analyzed as in the case of the preceding experiments. The ether extract had a marked lævo-rotation corresponding to 0.2 gram of *l*- $\beta$ -oxybutyric acid.

(*g*). A similar experiment was carried out in which 3.0 grams of the inactive salt was injected intravenously. The result was practically identical with the preceding experiment but the acetone excretion was rather more marked.

(*h*). An experiment was made in which 8.0 grams of the inactive acid was administered subcutaneously as sodium salt to a dog weighing 6 kilos. The amount of  $\beta$ -oxybutyric acid excreted unchanged was small but had a marked lævo-rotation corresponding to about 0.25 gram of the lævo acid.

## ON THE DECOMPOSITION OF ACETO-ACETIC ACID BY ENZYMES OF THE LIVER. PART II.

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(Received for publication, June 23, 1910.)

In our previous publication<sup>1</sup> the question of the oxidation of  $\beta$ -oxybutyric acid to aceto-acetic acid by an enzyme in the liver was primarily considered.<sup>2</sup> In addition some observations were made upon the decomposition of aceto-acetic acid by the liver confirmatory of those of Embden and Michaud.<sup>3</sup> The main results of these experiments may be summarized as follows: An enzyme capable of decomposing aceto-acetic acid without acetone formation was found to be present in normal liver tissue. The action of the enzyme was not accelerated by blood and was readily and completely inhibited by heat. Clear aqueous extracts of liver were without effect, but on the other hand washed liver cells had a very pronounced action. The question of the products (other than acetone and carbon dioxide) of the decomposition of aceto-acetic acid was not fully solved. Direct estimation of the acidity of distillates failed to give definite evidence of the formation of acetic acid but the production of acetic acid was demonstrated when the digestive mixtures were subjected to a process consisting essentially in hydrolysis with 50 per cent sulphuric acid and oxidation of the resulting volatile acids with chromic acid.

In the present communication we wish to record the fact that we have satisfied ourselves that *the primary product of this action of the*

<sup>1</sup> This *Journal*, vi, p. 373, 1909.

<sup>2</sup> In the experiments referred to we observed the action of liver tissue and extracts upon optically inactive sodium  $\beta$ -oxybutyrate. In the meantime we have made similar experiments with the natural or lævo-compound and find that the results in the two series of experiments are practically identical.

<sup>3</sup> Hofmeister's *Beiträge*, xi, p. 332.

*liver enzyme upon aceto-acetic acid is not acetic acid but lævo-rotatory  $\beta$ -oxybutyric acid.* It is clear that  $\beta$ -oxybutyric acid when subjected to the process of analysis, namely heating with 50 per cent sulphuric acid followed by oxidation with chromic acid, would be converted successively into crotonic and acetic acids:



We were led to suspect the formation of  $\beta$ -oxybutyric acid from the results obtained by one of us from experiments upon the intravenous injection of sodium aceto-acetate<sup>1</sup>, in which it was found that considerable amounts of *l*- $\beta$ -oxybutyric acid were excreted in the urine (cf. preceding paper). Although our experiments are not as complete as we would wish to make them, we are led to publish our observations at this time since communications have recently appeared by Blum<sup>2</sup> and by Maase in which essentially the same results are arrived at.

The results of our experiments, taken in conjunction with our previous results on the oxidizing enzyme acting upon  $\beta$ -oxybutyric acid, indicate that the liver is provided with a mechanism capable of effecting by means of enzyme action the mutual interconversion of  $\beta$ -oxybutyric acid and aceto-acetic acid:



The reaction involving the oxidation of  $\beta$ -oxybutyric acid to aceto-acetic acid is accelerated by the addition of blood or oxyhæmoglobin. The reverse change, *i.e.*, the reduction of aceto-acetic acid to  $\beta$ -oxybutyric acid, is not accelerated by the addition of blood. This result is obviously what would be anticipated.

The details of our experiments are as follows: The livers of dogs were removed under ether anæsthesia and rapidly ground in a previously warmed grinding apparatus and transferred to weighed warmed glass flasks, some of which contained neutral sodium aceto-acetate, while in others an equivalent amount of salt solution was used for a blank experiment. An amount of warm 0.8 per cent salt solution was added equivalent to the weight of liver taken. The quantities were arranged so that the flasks were about three-

<sup>1</sup> *Journ. Amer. Med. Assoc.*, liv, p. 1441.

<sup>2</sup> *Münch. med. Woch.*, lvii. p. 683, No. 13,



quarters filled and care was taken to avoid excessive aeration of the mass. The sodium aceto-acetate was prepared as in our previous experiments. The flasks containing the digestive mixtures were transferred as rapidly as possible to a thermostat maintained at 39–40°.

The rate of reduction of the aceto-acetic acid is rapid at the commencement of the experiment but soon ceases so that prolonged incubation is unnecessary. At the end of about two to seven hours the digestion mixtures were boiled with water, filtered and the residue well washed with boiling water. The filtrates were concentrated on the water-bath to about 50 cc. and then again filtered and evaporated to a syrup. Phosphoric acid was then added and the mass well ground up with excess of plaster of Paris. The resulting powder was extracted with ether in a Soxhlet apparatus for about six hours. The ether residue was then dissolved in hot water and clarified by the addition of a little charcoal. The resulting solution was examined in the polarimeter and the amount of *l*- $\beta$ -oxybutyric acid was calculated from these readings. The "blank" experiments in which no sodium aceto-acetate had been added failed to show a measurable *l*ævo-rotation. The *l*ævo-rotatory solutions containing the  $\beta$ -oxybutyric acid were tested as follows with positive results: (a) oxidation to acetone by means of chromic acid; (b) conversion into crotonic acid (melting-point, 73°) by distillation with 50 per cent sulphuric acid. The crotonic acid was separated from the distillate by ether extraction. It is advisable to separate by distillation any volatile fatty acids from the  $\beta$ -oxybutyric before distilling with sulphuric acid. (c) Black's reaction with hydrogen peroxide based upon the oxidation of  $\beta$ -oxybutyric acid to aceto-acetic acid.

WEIGHT OF LIVER.	ACETO-ACETIC ACID ADDED AS Na SALT.	TIME OF INCUBATION.	<i>l</i> - $\beta$ -OXYBUTYRIC ACID FORMED.
grams.	grams.	hours.	gram.
187	2.0	5	0.33
377	4.0	5	0.61
240	2.0	7	0.44
240	2.0	7	0.25



# THE PRODUCTS RESULTING FROM THE PUTREFACTION OF FIBRIN BY CLOSTRIDIUM CARNOFOETIDUS, SALUS AND RAUSCHBRAND.

By FRANCIS H. McCRUDDEN.

(From the Laboratory of Comparative Pathology, Harvard Medical School.)

(Received for publication, May 16, 1910.)

The following paper contains an account of some preliminary investigations on the products of putrefaction of certain obligatory anaërobes. In previous investigations on bacterial putrefaction the object has been usually either the study of putrefaction by bacteria in general or of the differences between the action of anaërobes and aërobes. I have been interested more particularly in the differences in the putrefactive products resulting from the action of certain obligatory anaërobes with the hope that perhaps some of the differences might be of diagnostic value. *Clostridium carno-fætidus*<sup>1</sup> and *Rauschbrand*<sup>2</sup> were selected as representing widely differing types of anaërobes in the belief that the difference in the results of putrefaction, if any, would be greatest.

The method of study was briefly as follows: A mixture of fibrin, water and salts, carefully sterilized, was inoculated with a twenty-four hour culture of the bacteria and then incubated for twenty days at a temperature of 36° C. At the end of this period the mixture, together with the gases formed, was subjected to chemical analysis. A check experiment in which the medium was not inoculated was also carried out. The details of the method follow.

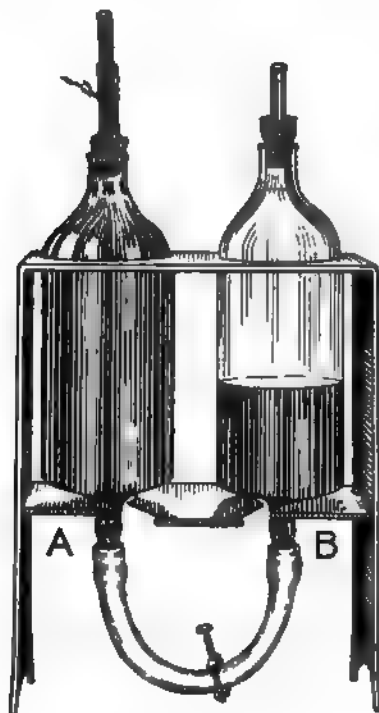
*The Apparatus.* The apparatus in which the incubation was carried out was devised and first described by Theobald Smith in

<sup>1</sup> This *Clostridium* culture was received from Kral's collection.

<sup>2</sup> This *Rauschbrand* culture was isolated by Dr. Smith from a case of the disease in Massachusetts.

## 110 *Clostridium* *Carno-foetidus* and Rauschbrand

1899<sup>1</sup> and slightly modified by me. It consists of two glass vessels each holding about two liters. Each vessel was drawn out at the bottom into the form of a bent glass tube and provided with an opening at the top for a stopper. The bent glass tubes of each pair of vessels were connected below by a short flexible rubber tube. Each bottle was provided above with a one-hole



rubber stopper through which passed a glass tube. In the bottle in which the putrefactive changes were to go on, one end of the glass tube which passed through the stopper was flush with the bottom of the stopper. The upper end of the same tube was connected to a second glass tube with a short rubber tube but separated from the second tube by a short glass rod inside the rubber

<sup>1</sup> *Journal Boston Soc. Med. Sciences*, 1899, p. 340.

tube. The object of this arrangement was to enable me to retain gases formed during the putrefaction and to collect them afterward. When the rubber tube was tied around the rod no gas could escape. When the tie was cut the rubber tube could be squeezed so that the gas could escape around the rod into the second tube. Bottle B was simply to relieve pressure in A and to receive fluids driven out by the accumulating gases. The tube through the stopper of B was closed only by a wad of cotton. The stand, made of tin or copper, which served to hold the bottles, is shown in the figure.

*The Culture Medium.* The culture medium was prepared as follows: To 1800 grams of finely divided impure fibrin from horse blood were added 600 c.c. of a solution containing 0.5 per cent sodium dihydrogen phosphate, 0.5 per cent sodium chloride and 10 cubic centimeters of a 15 per cent solution of potassium hydroxide. This solution was amphoteric to litmus paper. It was boiled with constant stirring to coagulate albumin, then allowed to cool and the solid material squeezed free from adherent liquid. 450 cubic centimeters of this liquid and 330 grams of the solid were used in each test culture.

*Sterilizing and Inoculating.* The culture medium was placed in the bottle and the stopper laid on top. The apparatus was placed in an autoclave and kept for 20 minutes at a temperature of 110° C. When cool, bottle B was elevated so that the culture medium filled A. The stoppers were then placed in the bottles, tied down and sealed with paraffine. The mixture was then inoculated through the tube in B, with precautions against contamination, by adding some of the culture from a long capillary tube. The conditions in A were anaërobic.

*Investigation of the Products.* The gas formed was allowed to escape into a gasometer, and then shaken successively with solutions of mercury cyanide, potassium hydroxide, and dilute sulphuric acid to dissolve respectively hydrogen sulphide and mercaptan, carbon dioxide, and ammonia.

200 cubic centimeters of the solution<sup>1</sup> were taken, made slightly

<sup>1</sup> These methods do not differ essentially from those used by Rettger: *This Journal*, ii, p. 71, 1906.

## 112 *Clostridium* *Carno-foetidus* and Rauschbrand

acid with sulphuric acid, warmed to 40° C. Air was drawn through it for two hours by means of a suction pump. The air from the flask, containing any hydrogen sulphide and mercaptan, was passed through a 3 per cent solution of cyanide of mercury and then through a solution of potassium hydroxide. The cyanide solution served to retain the hydrogen sulphide and mercaptan as sulphide and mercaptide of mercury, and the potassium hydroxide to retain any hydrocyanic acid set free.

The precipitate of sulphide and mercaptide of mercury was weighed. It was then dissolved in a little sulphuric acid and the gas set free passed into a solution of isatin in concentrated sulphuric acid to test for mercaptan.

After driving out the hydrogen sulphide and mercaptan, the solution was distilled with steam until 400 cubic centimeters of distillate was obtained. This distillate was made alkaline with sodium hydroxide and again distilled with steam until 250 cubic centimeters distillate was obtained. This second distillate was tested for indol and skatol.<sup>1</sup>

After distilling off the indol and skatol, the residue, containing any phenol, was made nearly neutral with hydrochloric acid saturated with carbon dioxide, and then distilled with steam until 250 cubic centimeters of distillate was obtained. This distillate was tested for phenol with Millon's reagent and with bromine water.

The residue from the first distillation was filtered, concentrated to small bulk and extracted with ether. The ethereal extraction was evaporated to dryness and the residue extracted with water. The aqueous solution was tested for aromatic oxy-acids and skatol-carbonic acid with Millon's reagent and acid ferric chloride respectively.

The solution remaining after extracting the aromatic oxy-acid and skatol-carbonic acid was concentrated and examined microscopically for leucin and tyrosin.

To the mother liquor from the leucin and tyrosin were added 5 volumes of 95 per cent alcohol to precipitate albumin and peptone. The precipitate, after washing with alcohol, was dissolved in water and tested for albumin and peptone.

<sup>1</sup> For tests see Salkowski's *Prakticum*, ed. of 1900, pp. 229-230.

The alcoholic filtrate was concentrated, acidified with acetic acid, and shaken with 5 cubic centimeters of bromine water to test for tryptophan.

## RESULTS.

	CLOSTRIDIUM CARNO- FOETIDUS	RAUSCHBRAND
Reaction to litmus.....	Alkaline.....	
Amount of gas.....	300 cubic centimeters	20 c.c.
Carbon dioxide.....	35 per cent.....	None
Hydrogen sulphide.....	15 per cent.....	None
Mercaptan.....	None.....	None
Ammonia.....	25 per cent.....	None
Residue (Hydrogen and methane).....	25 per cent.....	None
Indol .....	None.....	None
Skatol.....	None.....	None
Phenol.....	None.....	None
Hydrogen sulphide in solution weighed as mercury sulphide...	0.202 gram.....	None
Mercaptan in solution.....	None.....	None
Aromatic oxy-acids.....	Strong tests.....	None
Indolacetic.....	None.....	None
Albumin.....	Slight test.....	None
Peptone.....	None.....	None
Tyrosin .....	Present.....	None
Leucin.....	Present.....	None
Tryptophan.....	Present.....	Present

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It will be seen that there are distinct differences in the products of putrefaction in the two cases, differences which make it seem worth while for us to follow the matter further with the hope of making them of diagnostic value.



# THE METABOLISM OF SOME PURINE COMPOUNDS IN THE RABBIT, DOG, PIG, AND MAN.

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(Received for publication, May 20, 1910.)

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The study of the metabolism of the purine compounds has in recent years been directed most intensively toward the investigation of their behavior with extracts of organs and tissues. The existence of a series of enzymes capable of reacting upon various purines, and quite specific in their activities, has now become definitely established; and numerous researches have indicated that the distribution of these enzymatic properties is strangely varied in respect to the species as well as the organs of the individual. With the growing appreciation of this unlike equipment of different organisms as regards the purine-transforming enzymes,<sup>1</sup> it has been hoped to explain some of the peculiarities which are presented

<sup>1</sup> A useful summary of the present status of the investigation of these enzymes will be found in the paper by Wells: *This Journal*, vii, p. 171, 1910.

in the purine metabolism of different species. Thus one naturally correlates the occurrence of allantoin as an end product of purine metabolism in certain animals, and its absence in other species (*e.g.*, man), with a corresponding presence or lack of uricolytic enzyme. Similar considerations have been brought to apply to so-called guanine gout. This method of investigation has contributed in no small degree to a better understanding of the intermediary changes which the purines may undergo in living organisms; but its limitations must be frankly admitted. The indications derived from the study of tissue extracts need to be supplemented by metabolism experiments on the living individual where alone the ensemble of reactions involved manifest themselves in their appropriate relations.

Researches of the type just alluded to have by no means been wanting; but they have as a rule partaken of a qualitative character and have been concerned preëminently with the question of uric acid and allantoin formation. Valuable facts have been suggested, as, for example, the presumable absence of purine synthesis in the adult mammal, and the exclusion of the intestine as an organ for excretion of purines.<sup>1</sup> But an adequate metabolic history of all the individual purines which are commonly associated with physiological problems remains to be written. This is true, above all, in reference to the *quantitative* studies in metabolism which are called for to supplement the abundance of enzyme data now available. Guided by such considerations, we have undertaken experiments to determine to what extent and along what lines the familiar purines are transformed in the animal body.

The choice of a suitable method of administering the purines is not without difficulties. The oral path carries with it uncertainty regarding the absorption of the compounds fed and the possibility of a destructive loss through the agency of organisms in the alimentary tract. On the other hand, the parenteral introduction may be attended with untoward results which do not obtain where the purines enter the portal circulation by a slower process of alimentary absorption.<sup>2</sup> Despite these limitations, both methods

<sup>1</sup> Cf. Schittenhelm: *Zeitschr. f. exper. Pathol.*, iv, p. 766, 1907.

<sup>2</sup> Schittenhelm (*Zeitschr. f. physiol. Chem.*, lxii, p. 87, 1909) insists, perhaps on the basis of rather extreme conditions, on the inadvisability of drawing conclusions regarding normal process from the outcome of subcutaneous and intravenous injection trials with nucleic acid.

were employed by us, the parenteral paths of introduction being adopted in animals, while in man the purines were fed.<sup>1</sup> The analytical methods used were as follows:—the Kjeldahl-Gunning method, for *total nitrogen*; the Krüger-Schmid process,<sup>2</sup> for *uric acid* and *urinary purines*; the method of Krüger and Schittenhelm,<sup>3</sup> for *fecal purines*; titration with uranium acetate solution, for *phosphorus*; Folin's methods, for *urea*, *ammonia*, *creatine* and *creatinine*. The rabbits were fed 300 grams of carrots daily; on this diet they remain in apparently good condition for some time and excrete almost no uric acid. The urine was collected in daily periods under toluene, the last portion being expressed from the bladder at the same hour each day. The purine determinations were made on the fresh urines.

#### THE METABOLISM OF PURINES IN THE RABBIT AND THE DOG.

##### *Uric Acid.*

Increasing evidence has accumulated to show that uric acid is not the conspicuous end-product of purine metabolism in a number of animal species investigated, but that man forms a noteworthy exception in this regard.<sup>4</sup> Although allantoin appears to be the compound in the form of which the purine nitrogen is excreted in the dog, cat, and rabbit, for example, in man this substance is never found in the urine in more than minutest traces.<sup>5</sup> The relatively insignificant quantities of the purine bases in the urine of all these species may ordinarily be neglected in any consideration of their metabolic fate.

The best indication of the comparative resistance of uric acid to oxidation in man is derived from quantitative observations on the re-excretion of this substance after parenteral introduction of it,

<sup>1</sup> The experimental data are taken from the Dissertation of Dr. Lyman, Yale University, 1909.

<sup>2</sup> Krüger and Schmid: *Zeitschr. f. physiol. Chem.*, xlv, p. 1, 1905.

<sup>3</sup> Krüger and Schittenhelm: *Zeitschr. f. physiol. Chem.*, xlv, p. 14, 1905.

<sup>4</sup> Cf. Wiechowski: *Beitr. z. chem. Physiol. u. Pathol.*, xl, p. 109, 1908.

<sup>5</sup> Cf. Wiechowski: *Biochemische Zeitschrift*, xix, p. 368, 1909; xxv, p. 431, 1910; Schittenhelm and Wiener: *Zeitschr. f. physiol. Chem.*, lxiii, p. 283, 1909.

subcutaneously or intramuscularly.<sup>1</sup> In the six human experiments on record the amount of injected uric acid recovered in the urine ranged from 48 to 98 per cent.

In other species the few recorded observations afford quite different results. In dogs the increased output of uric acid after its parenteral introduction has never been reported higher than 21 per cent.<sup>2</sup> For rabbits the figures are: 16 per cent,<sup>3</sup> 5.6 per cent,<sup>4</sup> 18 per cent,<sup>5</sup> and 11 to 17 per cent.<sup>6</sup> We shall present the protocols of our own experiments because they have, in addition, a bearing on the question of whether the fraction of uric acid that escapes destruction is a constant one for any species. This so-called "integrative factor" of Burian and Schur was fixed by them for the rabbit at one-sixth of the uric acid which actually reaches the circulation.

The uric acid used was a Kahlbaum preparation twice recrystallized by Horbaczewski's method from concentrated sulphuric acid. Immediately before injection it was dissolved in a minimal quantity of normal sodium hydrate solution and made approximately isotonic with blood serum by addition of physiological saline solution. The solution was introduced slowly into the marginal ear vein in the rabbits, about 20 minutes being taken in each experiment. None of the animals died. That the injections were by no means always inert is indicated by the occasional finding of protein in the urine.<sup>7</sup>

<sup>1</sup> Cf. Burian and Schur: *Arch. f. d. ges. Physiol.*, lxxxvii, 326, 1901; Ibrahim and Soetbeer: *Zeitschr. f. physiol. Chem.*, xxxv, p. 1, 1902; Wiechowski: *Arch. f. exp. Pathol. u. Pharmakol.*, lx, p. 185, 1909; Benzúr: *Zeitschr. f. exper. Pathol.*, vii, p. 339, 1909.

<sup>2</sup> Burian and Schur: *loc. cit.* (4 to 12 per cent); Wiechowski: *Beitr. z. chem. Physiol.*, xi, p. 117, 1908 (21 per cent).

<sup>3</sup> Burian and Schur: *loc. cit.*, p. 325 (subcutaneous injections).

<sup>4</sup> Wiechowski: *Beitr. z. chem. Physiol.*, xi, p. 120, 1908 (subcutaneous injections).

<sup>5</sup> Bendix and Schittenhelm: *Zeitschr. f. physiol. Chem.*, xlii, p. 461, 1904 (subcutaneous and intravenous injections).

<sup>6</sup> Croftan: *Arch. f. d. ges. Physiol.*, cxxi, p. 377, 1908 (intravenous injections and diuresis).

<sup>7</sup> Cf. Starkenstein: *Arch. f. exp. Pathol. u. Pharm.*, lvii, p. 27, 1907.

INTRAVENOUS INJECTIONS—RABBITS.

WEIGHT OF RABBIT	DAILY URINE VOLUME	URINE NITROGEN PER DIEM			PHOS- PHORUS	DOSAGE, ETC., OF INJECTED URIC ACID
		TOTAL	URIC ACID	PURINE BASES		
gm.	cc.	gm.	gm.	gm.	gm.	
I. 2300	310	.312	....	....	....	
	330	.441	....	....	....	
	290	.319	.022	.000	....	.24 gm. uric acid = .08 gm. N.
II. 1600	210	.364	....	....	....	
	310	.385	....	....	....	
	300	.426	....	....	....	
	100	.339	.076	.003	....	1.0 gm. uric acid = .333 gm. N.
	180	.710	.000	.000	....	
	265	.656	.000	.000	....	
	160	.655	....	....	.061	
	250	.632	....	....	.091	
	230	.681	....	....	.079	
	250	.267	.019	.002	.006	.467 gm. uric acid = .156 gm. N.
III. 1900	110	.308	....	....	.010	
	155	.496	....	....	.041	
	260	.662	.047	.002	.010	.5 gm. uric acid = .167 gm. N.
	180	.757	.063	.003	.046	.5 gm. uric acid = .167 gm. N.
	225	.733	.054	.000	.032	.5 gm. uric acid = .167 gm. N.
	225	.733	....	.000	.032	Urices combined for analysis
	260	.723	.000	.000	.021	
	300	.532	.055	.006	.016	.5 gm. uric acid = .167 gm. N.
	220	.603	.010	.000	.010	.5 gm. uric acid = .167 gm. N.
	220	.603	....	.000	.010	Urices combined for analysis.
	220	.603	....	.000	.010	Protein present.
	190	.611	....	....	.029	
	205	.437	....	....	.026	
	210	.443	....	....	.036	

INTRAPERITONEAL INJECTIONS—RABBITS.

WEIGHT OF RABBIT	DAILY URINE VOLUME	URINE NITROGEN PER DIEM			PHOS- PHORUS	DOSAGE, ETC., OF INJECTED URIC ACID
		TOTAL	URIC ACID	PURINE BASES		
gm.	cc.	gm.	gm.	gm.	gm.	
IV. 2300	310	.312	....	....	.021	Rabbit used for experiment I. 1.0 gm.uric acid = 0.333 gm.N. Ate very little for several days after injection.
	330	.441	....	....	.042	
	...	....	....	....	....	
	200	.268	.034	.000	.014	
	82	.264	.009	.000	.006	
	160	.729	.003	.000	.032	
	220	.625	.000	.000	.041	
	220	.444	.000	.000	.060	
V. 1600	265	.656	.000	.000	....	1.0 gm.uric acid = 0.333 gm.N. Ate nothing for several days after injection.
	17	.083	.006	.000	....	
	70	.420	.009	.000	....	
	165	1.026	.000	.000	....	
	145	1.202	.000	.000	....	

SUMMARY OF INJECTION TRIALS WITH URIC ACID—RABBITS.

		URIC ACID NITROGEN			
		INJECTED	EXCRETED		
		<i>gms.</i>	<i>gms.</i>	<i>per cent.</i>	
Intravenous Injections	{	.080	.022	27	{
		.333	.076	23	
		.156	.019	12	
		.167	.047	28	
		.167	.063	38	
		.167	.054	32	
		.167	.055	33	
		.167	.010	6	
Intraperitoneal injections.	{	.333	.046	14	{
		.333	.015	4	
					Average = 25 per cent.
					Average = 9 per cent.

*Discussion*.—An inspection of the protocols shows that a *large proportion of the uric acid introduced in the rabbit does not reappear as such*. The quantity recovered is by no means a constant one; nor would one expect it to be under the variable conditions of concentration, etc., which must exist in such trials. The elimination of the unchanged rest was always rapid after the intravenous injections, never extending into the second day. After intraperitoneal injections there was greater delay in the elimination. Undoubtedly the uric acid reaches the circulation less effectively in these cases. Almagia<sup>1</sup> found that uric acid could frequently be detected in the cartilage; and Ebstein and Nicolaier<sup>2</sup> also state that it does not so readily leave the peritoneal cavity, being precipitated in part. It is further noteworthy that in some cases—not all—the injection of uric acid was accompanied by a fall in the total urinary nitrogen output, even when the food intake was constant. The urinary phosphorus showed the same fluctuations. Perhaps this is referable to some impairment of the kidneys, suggested especially by the occasional appearance of albuminuria.

### *Guanine.*

There are few satisfactory experiments in physiological literature on the metabolism of guanine. The conflicting data—mostly negative—reported from feeding experiments are scarcely convincing, owing to the comparative insolubility of the compound and the uncertainty regarding its absorption. Schittenhelm and Bendix<sup>3</sup> alone obtained clear-cut results. They injected solutions of guanine subcutaneously and intravenously into rabbits and observed an increased output of both uric acid and purine bases in the urine. Xanthine was identified among the latter. Unchanged guanine did not reappear.

We have conducted a number of trials. The guanine was in part procured from C. F. Boehringer und Söhne; in part prepared from glands. The latter product contained 46.1 per cent nitrogen (calculated: 46.4 per cent). In making the injections the

<sup>1</sup> Almagia: *Beitr. z. chem. Physiol.*, vii, p. 471, 1906.

<sup>2</sup> Ebstein and Nicolaier: *Virchow's Archiv*, clxxiii, p. 337, 1896.

<sup>3</sup> Schittenhelm and Bendix: *Zeitschr. f. physiol. Chem.*, xliii, p. 365, 1905.

suggestions of Schittenhelm and Bendix were followed. The animals stood the injections well, although quite strongly alkaline solutions were required to keep the guanine in solution. In rabbits the intravenous injections were made slowly into a marginal ear

INTRAVENOUS INJECTIONS—RABBITS.

WEIGHT OF RABBIT	DAILY URINE VOL- UME	URINE NITROGEN PER DIEM					DOSAGE, ETC., OF INJECTED GUANINE
		TOTAL	URIC ACID	PURINE BASES	CREATININE	CREATINE	
gm.	cc.	gm.	gm.	gm.	gm.	gm.	
VI. 2350	380	2.16	.006	.006			.089 gm. guanine = .041 gm. N.
VII. 1500	215	1.04	.024	.046			.445 gm. guanine = .206 gm. N.
	290	0.61	.008	.010			
	240	0.57	.000	.000			
	175	1.06	.003	.004			
VIII. 2000	275	0.59	.018	.013			.5 gm. guanine = .232 gm. N.
	200	0.82	.000	.000			
	200	0.86	.000	.000			
	250	0.79	.000	.001			
	235	0.64	.000	.001			
	200	0.69	.020	.010			.5 gm. guanine = .232 gm. N.
	165	0.76	.004	.001			
	170	0.80	.003	.002			
	180	0.79	.003	.002			
	265	.545	.004	.003	.026	.001	
IX. 2000	240	.446	.000	.002	.023	.001	
	310	.618	.032	.021	.013	.000	.5 gm. guanine = .232 gm. N.
	210	.486	.003	.002	.027	.003	
	250	.516	.004	.002	.021	.006	
	250	.468	....	.002	.028	.000	
	190	.426	.003	.002	.025	.000	
	235	.384	....	.003	.025	.000	
	225	.420	.002	.001	.028	.000	
	180	.54	.000	.000	.036	.000	
	195	.44	....	....	.036	.000	
X. 2400	310	.69	.025	—*	.039	.000	.5 gm. guanine = .232 gm. N.
	175	.49	.000	.001	.036	.000	
	250	.62	.000	.000	.038	.000	

\* Not determined: see Discussions.



INTRAPERITONEAL INJECTIONS—RABBITS.

RIGHT OF RABBIT	DAILY URINE VOLUME	URINE NITROGEN PER DIEM					DOSAGE, ETC. OF INJECTED GUANINE
		TOTAL	URIC ACID	PURINE BASES	CREATIN- INE	CREATINE	
gm.	cc.	gm.	gm.	gm.	gm.	gm.	
I. 1700	170	.43	....	....	....	....	.5 gm. guanine = .232 gm. N.
	230	.57	....	....	....	....	
	275	.59	....	....	....	....	
	225	.71	.000	.000	....	....	
	310	.68	.000	.000	....	....	
	260	.76	.000	.000	....	....	
I. 2400	250	.64	.000	.000	....	....	.8 gm. guanine = .3 gm. N.
	175	.49	.000	.001	.036	.000	
	250	.62	.000	.000	.038	.000	
	175	1.02	.008	.000	.040	.005	
	210	.69	.000	.000	.038	.000	
	210	.46	.000	.000	.032	.000	

SUMMARY OF THE INTRAVENOUS INJECTION TRIALS WITH GUANINE IN RABBITS.\*

NITROGEN INJECTED AS GUANINE	NITROGEN ELIMINATED AS		
	URIC ACID		INCREASE IN PURINE BASES
	gm.	per cent	gm.
.089	.006	8	.006
.206	.032	19	.056
.232	.018	10	.013
.232	.020	11	.010
.232	.032	17	.021
.232	.025	13	....

Average.....13

\* In computing the per cent excreted as uric acid, the fact that guanine contains one amino group which is split off as ammonia, and that thus one-fifth of the nitrogen of the guanine is not concerned in uric acid formation, is taken into consideration. For instance, in experiment VIII, 0.5 gram guanine was injected. Five-tenths of a gram guanine contains .232 gram nitrogen. Only four-fifths of this—or 0.186 gram nitrogen—is contained in the purine nucleus and can therefore, according to the accepted idea, be transformed to uric acid in the body. The percentages are computed on this basis.

vein during about 15 to 20 minutes. As the animals frequently refused to eat the carrots for several hours afterwards, it was found advisable to feed them just prior to the injections. The food intake was thus kept constant. The dogs were fed on a purine-free diet of bread and milk with bone meal to give a dry texture to the fæces. The injections were made into the external jugular vein with aseptic precautions, during urethane-ether narcosis.

### *Intravenous Injections—Dogs.*

XIII. A dog weighing 9 kgm. received 2 gm. guanine in 150 cc. solution. The output of uric acid nitrogen was 0.006 gm.; that of purine base nitrogen was 0.009 gm., representing an increase of about 0.006 gm. over the daily amount in the after period. The excretion of allantoin was notably increased, so that a gram or more could be crystallized out of the urine. M. p. 214°. The volume of the urine was considerably increased and protein was present on the day of the injection.

XIV. A dog weighing 6.5 kgm. received 2 gm. guanine intravenously as in the above experiment. The uric acid nitrogen output on the subsequent day was 0.007 gm. without any notable increase in the elimination of other purines. Over a gram of allantoin was obtained by crystallization. Protein appeared in the urine.

*Discussion*;—In the *dogs* guanine evidently experienced a ready conversion through the usual stages obtained in experiments with tissue extracts. That allantoin was the end product of this metabolism is made probable by the readiness with which it could be separated in large amounts by simple crystallization, where this method failed in the periods before and after the guanine administration. Since uric acid could not be separated in these other periods the appearance of the small fraction of the purine nitrogen in this form marks it as a probable intermediary product. With the *rabbits* the results correspond with those reported by Schittenhelm and Bendix. An attempt was made with the urine of experiment X to identify the purine compounds in the urine. No guanine, adenine or hypoxanthine could be detected; but 0.033 gram of a substance with the properties of xanthine was separated. It is thus probable that the *xanthine and uric acid represent eliminated fragments of the intermediary metabolic products of guanine introduced parenterally.*

Adenine.

Minkowski<sup>1</sup> called attention to an inflammatory condition of the kidneys which he observed after administration of adenine to dogs. He failed to obtain the large output of allantoin which characterized the ingestion of some of the other purine compounds. Nicolaier<sup>2</sup> identified the deposits found in the kidneys after subcutaneous injections of adenine in rats, as 6-amino-2,8-oxy-purine: and Ebstein and Bendix<sup>3</sup> obtained the same substance under similar conditions in rabbits. Quantitative data relating to the parenteral administration of adenine are not available. We have

INTRAVENOUS INJECTIONS—RABBITS

WEIGHT OF RABBIT	DAILY URINE VOLUME	URINE NITROGEN PER DIEM				DOSAGE, ETC., OF INJECTED ADENINE
		TOTAL.	URIC ACID	PURINE BASES	CREATIN- INE	
gm.	cc.	gm.	gm.	gm.	gm.	
XV.2000	240	510	.002	.002	.030	.5 gm. adenine = .295 gm. N.
	265	456	.002	.000	.030	
	260	714	.009	.090	.024	
	260	468	.001	.001	.029	
	225	480	.001	.001	.028	
XVI.2000	180	750		.004	.035	.5 gm. adenine = .295 gm. N.
	250	672	.000	.002	.036	
	150	354	.003	.012	.018	
	125	636	.000	.001	.027	
	190	972	.000	.001	.035	
	250	772			.029	
	225	756	.000	.002	.028	
XVII.240	300	866	.008	.074	.027	.5 gm. adenine = .295 gm. N.
	225	702	.000	.002	.030	
	255	648	.000	.000	.026	
	265	632	.000	.001	.033	
	260	790	.013	.076	.037	
	180	540	.000	.000	.036	
	195	440			.036	

<sup>1</sup> Minkowski: *Arch. f. exper. Pathol. u. Pharmacol.*, xli, p. 375, 1898.  
<sup>2</sup> Nicolaier: *Zeitschr. f. klin. Med.*, xlv, p. 359, 1902.  
<sup>3</sup> Ebstein and Bendix: *Virchow's Archiv*, clxxviii, p. 464, 1904.

INTRAPERITONEAL INJECTIONS—RABBITS

WEIGHT OF RABBIT	DAILY URINE VOLUME	URINE NITROGEN PER DIEM				DOSAGE, ETC., OF INJECTED ADENINE
		TOTAL	URIC ACID	PURINE BASES	CREATIN- INE	
gm.	cc.	gm.	gm.	gm.	gm.	
XVIII.2000	125	.636	.000	.001	.027	
	190	.972	.000	.001	.035	
	125	.900	.000	.017	.032	.5 gm. adenine = .295 gm.N.
	80	.570	.000	.003	.029	Ate nothing.
	65	.774	.000	.002	.032	Ate 100 gm. carrots.
	190	1.158	.003	.001	.034	Ate 230 gm. carrots.
	230	.570	....	....	.035	
XIX.1800	250	.684	....	....	.036	
	230	.850	.003	.011	.037	.5 gm. adenine = .295 gm.N.
	230	.816	....	....	.032	
	190	.664	....	....	.033	

made experiments on rabbits and dogs. The adenine used was prepared from thymus glands, being separated first as the picrate. On analysis it yielded 51.4 per cent of nitrogen (calculated: 51.8 per cent). It was not necessary to make the solutions for injection as strongly alkaline as in the case of guanine, since adenine does not separate out as readily. The animals withstood the injections well; in the rabbits an effect on respiration was usually noted. The after effects appeared to be more lasting than with the guanine, judging by the fact that, in contrast with the guanine rabbits, the adenine animals in some cases refused food for several days after the injections.

*Intravenous Injections—Dogs.*

The injections were made under anæsthesia, as described for guanine.

XX. A dog weighing 5.2 kgm. received 100 cc. containing 1 gm. adenine. Uric acid could not be separated from any of the urines, and no allantoin crystallized out. Following the injection, the output of purine bases was markedly increased, 0.24 gm. adenine picrate (m. p. 277°) being separated.

XXI. A dog weighing 9 kgm. received 1 gm. adenine as in Experiment XX. The purines on the injection day were much increased. No uric acid could be separated. Adenine was recovered as the picrate, 0.045 gram being separated (melting point—275°). The filtrate from the adenine pi-

crate still contained some purine. This was precipitated with copper sulphate-sodium bisulphite and the resulting precipitate found to contain 0.008 gram of nitrogen. The characteristic crystals of allantoin separated in small quantity from the urine of the day after the injection.

Albuminuria was noted in both dogs after the injections.

*Discussion:*—The phenomena observed in both dogs and rabbits, viz., little uric acid and allantoin, with relatively large output of unaltered adenine after adenine injection, contrasted with larger outputs of allantoin and intermediary xanthine and uric acid and small yield of bases after guanine administration, emphasize the comparative resistance of adenine to metabolic change in these species. It may be more than a corresponding coincidence that the occurrence of guanase is more widespread in rabbit and dog tissues than that of adenase. Similar differences have marked feeding experiments with the two purines.<sup>1</sup> Qualitatively, however, the metabolic changes are comparable in the two cases. It is interesting to note that the smaller quantity of adenine was recovered from the larger animal, as might be expected after the same dose.

### *Hypoxanthine.*

Burian and Schur<sup>2</sup> have reported experiments on the parenteral introduction of hypoxanthine. An intravenous injection was found by them to be unsatisfactory (l. c., p. 291). The subcutaneous injections were followed by an increased output of urinary purine compounds in both dog and rabbit, the increase being confined to the uric acid fraction in the dog at least. Ebstein and Bendix<sup>3</sup> have reported the microscopic findings in the kidneys after intravenous injections of hypoxanthine in rabbits.

We can report one trial in which 0.69 gm. hypoxanthine (= 0.28 gm. N) was injected into a rabbit weighing 3 kilos. The urine of the following 24 hours, 500 c.c. in volume, contained no protein; 0.124 gm. uric acid (= 0.041 gm. N) was separated and 0.134 gm. purine base N determined. Hypoxanthine was the conspicuous base present, 0.094 gm. of the nitrate being separated and

<sup>1</sup> Cf. Schittenhelm: *Arch. f. exper. Pathol. u. Pharm.*, xlvii, p. 432, 1902; also Jones: *Zeitschr. f. physiol. Chem.*, lxxv, p. 383, 1910.

<sup>2</sup> Burian and Schur: *Archiv f. d. ges. Physiologie*, lxxxvii, p. 239, 1902.

<sup>3</sup> Ebstein and Bendix: *Virchow's Archiv*, clxxviii, p. 464, 1904.

identified ( $N = 32.36$  per cent; calculated for  $C_5H_4N_4O.HNO_2 + H_2O = 32.26$  per cent). A trace of substance resembling xanthine was separated, but allantoin could not be isolated by simple crystallization. In this experiment about 15 per cent of the nitrogen introduced as hypoxanthine was excreted as uric acid.

#### THE METABOLISM OF PURINES IN THE PIG.

Investigations by Schittenhelm and Bendix<sup>1</sup> have shown that the end products of purine metabolism in the pig are not qualitatively different from those found in the other familiar species. They were led to examine the urine of the pig because the occurrence of "guanine gout" in this animal, as well as the comparative deficiency of its tissues in certain of the purine-transforming enzymes, made it seem likely that variations from the usual type might be characteristic in this case. An analysis of urine from a pig fed upon bread, milk, and meat scraps, as well as urines taken from animals killed at the slaughter house, showed that the purine bases preponderate in amount over uric acid in the urine. Schittenhelm and Bendix isolated xanthine and hypoxanthine, together with smaller quantities of adenine and guanine.

Previous experience<sup>2</sup> with the enzymes of the tissues of the pig, characterized as they are by a deficiency in guanase and some other enzymes, suggested the desirability of additional data on the behavior of purines introduced into this species. A few experiments made on a pig about five months old and weighing 50 kgm. are reported below. The animal was kept in a metabolism cage and fed upon a purine-free diet of bread (1 kgm.), milk (3500 c.c.), and a little salt each day. The urine was collected under toluene. In as much as it was impracticable to obtain urine in exact 24 hour periods, the feeding trials are reported in periods of three to five days. The purines fed were dissolved in a minimal quantity of sodium hydrate solution and mixed with the food, which was given in two daily portions. Care was taken to wash the cage thoroughly so that the urine would not become contaminated with food purine.

<sup>1</sup> Schittenhelm and Bendix: *Zeitschr. f. physiol. Chem.*, xlviii, p. 140, 1906.

<sup>2</sup> Cf. Mendel and Mitchell: *Amer. Journ. of Physiol.*, xx, p. 81, 1907.

*Daily Analyses of Pig's Urine.*

THE ENDOGENOUS PURINES.

DATE		VOLUME	SPECIFIC GRAVITY	PURINE BASE N.	URIC ACID N.
		cc.		gm.	gm.
1909, V,	17	980	1.015	.055	.016
	18	1460	1.009	.021	.012
	19	3000	1.009	.027	.008
	20	3400	1.010	.041	.020
	21	2960	1.012	.038	.009
			Average	.036	.013

FEEDING OF HYPOXANTHINE NITRATE.

DATE	VOLUME	SPECIFIC GRAVITY	PURINE BASE N	URIC ACID N	PURINE FED
	cc.		gm.	gm.	
1909, V, 23	4250	1.010	.049	.026	1.0 gm. hypoxanthine nitrate = 0.258 purine N.
24	3800	1.010	.065	.011	
25	3200	1.012	.067	.018	
26	3200	1.012	.056	.012	
		Sum	.237	.067	

FEEDING OF ADENINE.

DATE	VOLUME	SPECIFIC GRAVITY	PURINE BASE N	URIC ACID N	PURINE FED
	cc.		gm.	gm.	
1909, V, 28	3660	1.011	.080	.009	0.92 gm. adenin = 0.38 gm. purine N.
29	3040	1.014	.065	.013	
30	4100	1.013	.047	.023	
		Sum	.192	.045	

## Metabolism of Purine Compounds

## FEEDING OF GUANINE.

DATE	VOLUME	SPECIFIC GRAVITY	PURINE BASE N	URIC ACID N	PURINE FED
	cc.		gm.	gm.	
1909, V, 31	4200	1.011	.075	.017	1.5 gm. guanine = 0.696 purine N.
VI, 1	3400	1.012	.107	.015	
2	980	1.016	.086	.022	
3	4680	1.007	.043	.014	
		Sum	.311	.068	

## FEEDING OF NUCLEOPROTEIN (PANCREAS).

DATE	VOLUME	SPECIFIC GRAVITY	PURINE BASE N	URIC ACID N	PURINE FED
	cc.		gm.	gm.	
1909, VI, 5	2740	1.014	.168	.044	1800 gm. pig's pancreas
6	1960	1.014	.078	.027	
7	3760	1.009	.041	.024	
8	4850	1.008	.064	.042	
		Sum	.351	.137	

\* The fresh pancreas was boiled and fed in three portions. It evoked diarrhoea, but the uri was not contaminated.

## FEEDING OF SODIUM URATE.

DATE	VOLUME	SPECIFIC GRAVITY	PURINE BASE N	URIC ACID N.	PURINE FED
	cc.		gm.	gm.	
1909, VI, 10	4800	1.006	.063	.050	5 gm. sodium urate = gm. N. 7 gm. sodium urate = gm. N.
11	4000	1.006	.050	.050	
12	3600	1.011	.056	.036	
		Sum	.169	.136	



**Discussion:**—These experiments confirm the observations of the *preponderance of purine bases over uric acid in the urine* even on a purine-free diet where the excretion was on an endogenous basis. We attempted to isolate the purines under these circumstances from the collected urine of three days. The yields were as follows: uric acid .07 gm. (recrystallized), xanthine .06 gm., hypoxanthine nitrate .04 gm. (some lost). Adenine and guanine could not be obtained in weighable amounts and must have been present, if at all, in far less conspicuous quantity.

Some idea of the effect of the purines fed upon the purine output in the urine can be obtained by comparing the calculated total endogenous output for each period with the actual excretion after administration of the various compounds reported. For example, on May 23 (when 1 gram of hypoxanthine nitrate [= 0.258 gm. purine N] was fed) and the three subsequent days, a total purine base N elimination of 0.237 gm. was recorded. The average daily endogenous output previously noted being 0.036 gm., the corresponding figure for four days is 0.144 gm. N. Accordingly the excess,  $0.237 - 0.144 = 0.093$  gm. N, may be attributed to the hypoxanthine. The absorption factor in these experiments was undetermined, but presumably not unfavorable at the dilutions in which the purines were fed. The calculated outcome of the purine feedings is summarized here.

INFLUENCE OF PURINE FEEDING ON PURINE ELIMINATION—FIG.

	PURINE N FED	PURINE N RECOVERED IN URINE AS	
		PURINE BASE N	URIC ACID N
	gm.	gm.	gm.
Hypoxanthine.....	.258	.092	.015
Adenine.....	.477	.084	.006
Guanine.....	.696	.166	.016
Uric Acid.....	2.83	.061	.097
Pancreas.....	....	.207	.098
Daily endogenous output.....		.036	.013

Assuming a satisfactory coefficient of absorption in these experiments, a large deficit of purine N appears in the table. Whether allantoin plays an important rôle as an end product, we are unable to say at present. It could not be obtained by the crystallization method even after several grams of allantoin itself were fed; but the reports of Wiechowski<sup>1</sup> for other mammals leave little dependence to be placed on the cruder method. Nevertheless it should be noted that this simple procedure, which regularly leads to the separation of allantoin in abundance after feeding uric acid to the pancreas to dogs and cats,<sup>2</sup> failed to furnish crystals after comparable trials with pig's urine.

Three-fifths of the urine obtained after the feeding of pancreas was examined for its purines. From it 0.085 gm. xanthine and 0.165 gm. hypoxanthine nitrate were isolated. Attention is called to the increased elimination of purine bases after administration of sodium urate. The quantity of uric acid recovered, such after the ingestion of 8.5 grams in two days is small and emphasizes the probability that *uric acid is not the chief end product of purine metabolism in the pig.*

#### THE METABOLISM OF PURINES IN MAN.

The deficiencies of the parenteral method of introduction in the study of purine metabolism have been referred to. Where possible it ought to be supplemented by feeding experiments. Results obtained with animals can no longer be applied without reserve to man since the striking differences in the enzyme equipment of the different species has been pointed out. We have, therefore, conducted systematic feeding experiments with the different purines on man. There are numerous related studies in the literature, but none, we believe, in which the experimental conditions have all been kept so constant and comparable. An attempt has been made to follow not only the fate of the individual purines, but also any disturbance which they might induce in other features of nitrogen metabolism.

The partition of nitrogen in the urine has been studied in the

<sup>1</sup> Wiechowski: *Biochemische Zeitschrift*, xix, p. 368, 1909; also xxv, p. 4, 1910, which appeared since this paper went to press.

<sup>2</sup> Cf. the data in Mendel and Dakin: *This Journal*, vii, p. 153, 1910.

subjects. One, J. F. L., 28 years of age, body weight = 68 kgm.: the other, W. W. H., 25 years of age, body weight = 55 kgm. The routine of analytical work involved, together with other work, called for rather more than ordinary activity from the two subjects, an activity which was about alike from day to day. A fixed and practically purine-free diet was taken, the time of eating and the habits of life being kept as nearly constant as possible from day to day. The diet was as follows:

*Breakfast at 8.30 a.m.*

Half a Grape Fruit, about.....	200 gm.
"Force," Breakfast Cereal.....	20 gm.
Banana .....	80 gm.
Milk.....	400 cc.
Sugar .....	60 gm.

*Dinner at 1 p.m.*

Potato, .....	100 gm.
2 Eggs, about.....	100 gm.
Bread .....	150 gm.
Butter .....	50 gm.
Milk .....	400 cc.
Pickle (Cucumber), about.....	30 gm.

*Supper at 6 p.m.*

Cream Cheese.....	20 gm.
"Uneda" Biscuit.....	25 gm.
Milk .....	500 cc.
Pickle, about.....	30 gm.
Orange, about.....	150 gm.

This diet was calculated to contain 13.2 grams of nitrogen and to yield about 2700 calories. The same diet was eaten by both subjects. J. F. L. lost about 1 kgm. in body weight during the 20 days of the experiment. W. W. H. maintained a constant body weight. The condition of health of both subjects was excellent throughout the experiment, no sickness or ill feeling being experienced at any time. The urine was collected in 24-hour periods in bottles containing toluene. The daily period was ended at 7:30 each morning, and analysis of the urines begun shortly afterwards.

The fæces were mixed with alcohol and a few drops of sulphuric acid immediately after collection and dried on the water bath. The fæces periods were marked off with lamp-black or carmine tablets with the proper meals.

The selected diet was started four days before analysis of the urines began. A three-days normal period was then obtained and the experimental periods followed. The purines administered were dissolved in a minimal amount of normal sodium hydroxide solution and a few c.c. of water. The resulting solution was mixed with the milk, one-third of the purine being taken with each meal on the experimental day. The purines used in all the experiments were prepared from glands. The adenine and guanine were parts of the same preparations used in the rabbit and dog experiments already reported. The xanthine was obtained as the free base and found to contain 36.8 per cent nitrogen; calculated for  $C_5H_4N_4$  = 36.9 per cent. The hypoxanthine was administered as the nitrate. It was found to contain 32.3 per cent nitrogen; calculated for  $C_5H_4N_4O.HNO_3 + H_2O$  = 32.26 per cent. The urines were acid to litmus throughout the experiment. No protein or reducing substances could be detected at any time. The summaries of the analyses follow:

W. W. H., 55 KGM.

DATE	NITROGEN ELIMINATED AS							PHOSPHORUS	SPECIFIC GRAVITY	VOLUME	PURINE FED
	TOTAL	UREA	CREATININE	AMMONIA	URIC ACID	PURINE BASES	UNDETERMINED				
1899	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	1.0+	cc.	
4, 18	11.52	10.31	.542	.346	.116	.014	.192	1.15	21	1220	
19	11.56	9.85	.537	.352	.119	.015	.687	1.21	20	1300	
20	11.83	10.15	.547	.352	.131	.017	.633	1.18	21	1220	
21	12.26	10.48	.537	.324	.245	.027	.647	1.18	27	1060	1.5 gm. hypoxan-
22	12.15	10.41	.542	.339	.208	.018	.633	1.19	24	1240	thine nitrate.
23	11.29	9.76	.542	.361	.153	.015	.459	1.18	26	960	
24	11.66	10.09	.537	.389	.138	.017	.489	1.14	22	1080	
25	11.53	9.95	.539	.313	.249	.020	.459	1.15	23	1400	1.0 gm. xanthine.
26	12.10	10.36	.537	.389	.195	.019	.600	1.13	29	1040	
27	11.88	10.17	.542	.518	.128	.012	.510	1.00	26	650	
28	12.51	10.74	.537	.367	.232	.024	.610	1.11	25	1060	1.5 gm. guanine.
29	12.10	10.29	.537	.324	.300	.035	.614	1.09	21	1300	1.5 gm. guanine.
30	11.21	9.66	.537	.389	.176	.019	.429	1.16	26	1000	
31	10.69	9.15	.531	.356	.135	.019	.499	1.08	23	1000	
32	10.31	8.90	.534	.308	.120	.015	.433	1.04	28	900	
33	11.37	9.65	.537	.367	.188	.030	.598	1.12	25	860	1.0 gm. adenine.
34	12.20	10.35	.540	.378	.182	.015	.735	1.16	20	1160	
35	12.02	10.31	.522	.384	.142	.010	.652	1.21	26	1000	
36	11.02	9.23	.531	.384	.137	.018	.720	1.03	24	1100	
37	10.75	9.28	.537	.367	.127	.014	.425	1.09	24	1040	

J. F. L., 68 Kgm.

DATE	NITROGEN ELIMINATED AS									PURINE
	TOTAL	UREA	CREATININE	AMMONIA	URIC ACID	PURINE BASES	UNDETERMINED	PHOSPHORUS	SPECIFIC GRAVITY	
1909	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	1.0+	cc.
II, 18	11.29	9.62	.608	.497	.117	.015	.433	1.04	17	1480
19	10.75	8.85	.623	.497	.114	.016	.650	1.05	25	900
20	11.69	9.92	.616	.503	.142	.016	.493	1.06	25	900
21	12.87	10.98	.616	.486	.222	.025	.541	1.17	24	1040 1.5 gm. :
22	12.50	10.44	.609	.579	.200	.014	.656	1.16	20	1240 thine :
23	11.77	9.95	.616	.572	.163	.015	.454	1.16	25	1040
24	12.85	10.93	.616	.568	.146	.015	.575	1.07	21	1300
25	12.07	9.80	.619	.518	.224	.017	.892	1.13	27	960 1.0 gm. x
26	13.12	11.02	.661	.540	.178	.019	.747	1.03	24	1120
27	12.17	11.34	.616	.572	.152	.017	.476	1.07	24	950
28	12.13	10.32	.616	.514	.183	.028	.469	1.01	28	820 1.5 gm. g
III, 1	13.12	11.24	.616	.475	.237	.031	.521	1.04	25	1160 1.5 gm. g
2	11.18	9.32	.616	.532	.181	.019	.482	1.04	28	800
3	10.95	9.06	.609	.551	.130	.015	.575	1.04	26	800
4	11.77	10.16	.620	.482	.129	.013	.366	1.05	23	1010
5	11.29	9.43	.614	.562	.167	.030	.487	1.02	19	1200 1.0 gm. ad
6	12.24	10.30	.616	.529	.182	.016	.597	1.10	16	1500
7	11.37	9.55	.616	.498	.161	.015	.530	1.16	32	710
8	12.61	10.49	.623	.577	.132	.017	.771	0.94	17	1380
9	10.48	8.44	.616	.529	.132	.013	.750	0.92	33	600

The endogenous uric acid output for J. F. L., determined taking an average of the 3 normal days and of the output on M 3, 4, 8 and 9, is 0.128 gram uric acid nitrogen. The endoge value for W. W. H. determined from an average of 3 normal and of the output on February 27, March 4 and 9, is 0.124 g uric acid nitrogen a day. On the 3 normal days J. F. L. excre an average of 0.016 gram purin base nitrogen in the urine W. W. H. 0.015 gram.

## SUMMARY OF THE RESULTS\*

PURINE FED		NITROGEN IN PURINE NUCLEUS FED	INCREASE IN NITROGEN ELIMINATED AS			
			URIC ACID		PURINE BASES	
		gm.	gm.	per cent	gm.	per cent
W. W. H.	Hypoxanthine.....	0.387	.248	64	.015	4
	Xanthine.....	0.369	.196	53	.009	2
	Guanine.....	1.114	.347	31	.037	3
	Adenine.....	0.414	.153	37	.015	3.6
J. F. L.	Hypoxanthine.....	0.387	.219	56.6	.009	2
	Xanthine.....	0.369	.170	46	.004	1
	Guanine.....	1.114	.217	19.5	.030	2.7
	Adenine.....	0.414	.126	30	.014	3

\* The percentages of increased uric acid and purine bases eliminated are computed on the basis of the nitrogen contained in the purine nucleus of the base administered.

The analyses show that all four purines produced a marked rise in urinary uric acid and a small, yet noticeable increase in elimination of purine bases. Of the purine ingested, W. W. H. excreted, in every case, a larger per cent as uric acid and purine bases than did J. F. L. It may be worthy of note in this connection that W. W. H. is a much smaller and lighter individual than the other subject, and hence may possess a more limited power for uric acid destruction.

In order to get evidence regarding a possible failure of absorption of the purines to account for the missing fraction, the fæces were analyzed for each period. The data are given below:

## ANALYSIS OF FÆCES.

PERIOD	J. F. L.		W. W. H.	
	WT. AIR DRY FÆCES PER DIEM	PURINE BASE N PER DIEM	WT. AIR DRY FÆCES PER DIEM	PURINE BASE N PER DIEM
	gm.	gm.	gm.	gm.
Normal.....	26	.073	25	.063
Hypoxanthine.....	17	.050	22.4	.082
Xanthine.....	30	.075	23.3	.066
Guanine.....	18	.068	20.9	.055
Adenine.....	21	.075	24.2	.058

The effect of the purines of meat (largely free hypoxanthine) on the elimination of purine compounds is well illustrated by the results of another series of unpublished experiments carried out by Dr. Hilditch in this laboratory. The same two subjects, J. F. L. and W. W. H., served for this experiment also. The diet was the same as in the purine experiments, except that 100 grams of meat were substituted for the eggs and part of the milk, the nitrogen and calories being kept practically constant. To effect this, the sugar and butter were increased over the amounts used in the purine experiments. The meat diet was eaten on four days before the urine was collected and the analyses made, so that the full effect of the meat on metabolism should have been produced by that time.

In the case of J. F. L. uric acid nitrogen was increased by 100 grams of meat per day from an average of 0.124 gram on a purine-free diet to 0.156 gram, or 0.032 gram. With W. W. H. after adding 100 grams of meat to the purine-free diet there was an increase in the uric acid nitrogen excreted from 0.122 gram a day to 0.153 gram, or 0.031 gram. According to Burian and Schur, 100 grams of meat contain 0.060 gram of purine nitrogen. Hence in the case of J. F. L. 53 per cent, and in the case of W. W. H. 51 per cent, of the purine nitrogen contained in the meat was excreted as uric acid.

Urine Analyses—Meat Diet.

J. F. L.

DATE		NITROGEN ELIMINATED AS					SPECIFIC GRAVITY	VOLUME		
		TOTAL	UREA	AMMONIA	URIC ACID	PURINE BASE				PHOSPHORUS
1909		gm.	gm.	gm.	gm.	gm.	gm.		cc.	
II,	18	11.29	9.62	.497	.117	.015	1.04	17	1480	Purine free diet.
	19	10.75	8.85	.497	.114	.016	1.05	25	900	
	20	11.69	9.92	.503	.142	.016	1.06	25	900	
III,	12	11.45	9.55	.550	.156	.015	.895		710	Same diet with 100 gm. meat substituted for eggs and milk
	13	12.96	10.98	.550	.156	.014	.972		760	
	14	12.91	10.77	.570	.156	.014	1.044		1000	



W. W. H.

DATE	NITROGEN ELIMINATED AS					PHOSPHORUS	SPECIFIC GRAVITY	VOLUME	
	TOTAL	UREA	AMMONIA	URIC ACID	PURINE BASE				
1900	gm.	gm.	gm.	gm.	gm.	gm.		cc.	
II, 18	11.52	10.31	.346	.116	.014	1.15	21	1220	} Purine free diet.
19	11.56	9.85	.352	.119	.015	1.21	20	1300	
20	11.83	10.15	.352	.131	.017	1.18	21	1220	
III, 12	11.93	10.05	.32	.150	.015	1.04		1100	} Same diet with 10 gm. meat substituted for eggs and milk
13	11.77	10.34	.41	.150	.014	1.02		880	
14	12.15	10.26	.38	.159	.015	1.03		980	

This increase is quite comparable with the figures obtained in the previous experiment with pure hypoxanthine and corresponds closely with that recorded by Burian and Schur.

*Discussion:*—Before commenting upon the results on man detailed above, it may be helpful to review briefly some of the views which have been entertained by others<sup>1</sup> regarding the origin of excreted uric acid and purine bases. Although extract of meat and hypoxanthine were generally observed to induce an increased excretion of uric acid, the few trials reported with the other free purine bases have given rather variable results. On the other hand, ingestion of nucleoproteins and nucleic acid complexes, which readily yield adenine and guanine, has long been known to be accompanied by increased output of uric acid, with little effect on the elimination of the other purines. Krüger and Schmid<sup>2</sup> attempted to solve the problems already raised in the minds of others, viz., whether the metabolism of the free purines proceeds along different lines when they are introduced in the more elaborate complexes of the nucleic acid molecule. They fed the four purine

<sup>1</sup> It is unnecessary to present a systematic account of these investigations, since the literature has been compiled to 1906 by Bloch: *Biochemisches Centralblatt*, v, 1906.

<sup>2</sup> Krüger and Schmid: *Zeitschr. f. physiol. Chem.*, xxxiv, p. 549, 1902.

bases to the same individual and observed the following change expressed in percentages of the ingested purine nitrogen eliminated as uric acid.

After feeding hypoxanthine.....	about 62 per cent
After feeding adenine.....	about 41 per cent
After feeding xanthine.....	about 10 per cent
After feeding guanine.....	very small

The change in the output of purine bases was rather small the largest observed being in the case of adenine, with an increase of 3 per cent. Krüger and Schmid concluded that the conversion of all of these bases to uric acid is a direct one, not associated with altered metabolism of leucocytes. Minkowski<sup>1</sup> and Burian and Schur<sup>2</sup> had already reported exogenous increases of uric acid corresponding to 46 and 48 per cent respectively of hypoxanthine fed by them.

Landau<sup>3</sup> fed hypoxanthine in quantities ranging from 0.8 to 1.5 grams and observed constant increases in the output of uric acid ranging from 46 to 88 per cent of the ingested purine. The variations thus noted led him to the conclusion that the elimination of exogenous uric acid is subject to individual variation independent of body weight. These inequalities, comparable with those which one finds for endogenous uric acid in different persons are attributed by Landau to fluctuations in the uricolytic equipment of the individual subject. He attempts, furthermore, to maintain that the further the purine compounds are removed chemically speaking, from uric acid the slower they yield uric acid to the circulation and the more complete the uricolysis becomes. On this basis xanthine and hypoxanthine should yield more exogenous uric acid than the amino-purines or nucleoproteins.

Brugsch and Schittenhelm<sup>4</sup> have reported the results of feeding free purines to a gouty individual. The exogenous uric acid attributable to these compounds was as follows:

Adenine.....	50 per cent
Guanine .....	trace
Hypoxanthine .....	13 per cent

<sup>1</sup> Minkowski: *Archiv für exper. Pathol. u. Pharmacol.*, xli, p. 375, 1898.

<sup>2</sup> Burian and Schur: *Archiv f. d. ges. Physiologie*, lxxxvii, p. 239, 1901.

<sup>3</sup> Landau: *Deutsches Arch. f. klin. Med.*, xcv, p. 280, 1909.

<sup>4</sup> Brugsch and Schittenhelm: *Zeitschr. f. exper. Pathologie*, v, p. 215, 190

Rotky<sup>1</sup> has studied the elimination of uric acid and purine bases after feeding guanine and hypoxanthine to patients of various types. Some of these exhibited diminished capacity for eliminating the purine derivatives; in others, the output of uric acid was notably increased after the administration of 1 gram of guanine hydrochloride or 0.5 gram of hypoxanthine, the increment being in some cases as high as the 40 per cent equivalent of the purine intake. The results on these pathological subjects differ from those reported by us in respect to the extreme variations shown in the different conditions. Thus in some cases the increment associated with the ingestion of these purines was confined almost entirely to the urinary purine bases which are scarcely increased under these conditions in the healthy subject. In others there was evidently poor absorption from the alimentary tract; and in still different cases only a small fraction of the intake of purine nitrogen could be accounted for in the form of immediate purine derivatives in the excreta. A few selected figures from Rotky's protocols will illustrate this:

PATHOLOGICAL CONDITION	PURINE FED	EXOGENOUS PURINE ELIMINATED IN PERCENTAGE OF INTAKE, IN		
		URINE		FÆCES
		URIC ACID N.	PURINE BASE N.	PURINE BASE N.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Myeloid leukaemia.....	hypoxanthine.....	39	4	18
Chronic alcoholism.....	guanine.....	25	3	1
Diabetes mellitus.....	guanine.....	0.6	72	4
Acute nephritis.....	hypoxanthine.....	I.	34	3
Chronic polyuria.....	guanine.....	24	36	27

The literature of this subject contains enough data to make clear that intermediary purine metabolism may be disturbed markedly in a number of pathological conditions in man, and that figures obtained by investigation of abnormal individuals can not be directly compared with those reported by us from the observation

<sup>1</sup> Rotky: *Deutsches Archiv f. klin. Med.*, xcvi, p. 540, 1910.

of healthy subjects. Our own experiments reported in the preceding protocols emphasize the fact that *all of the familiar purines may lead to an increase in exogenous uric acid in the urine of man with (quantitatively) little influence on the elimination of the purine bases*. They may be interpreted to support the most prevalent view that *uric acid is a stage in the metabolism of exogenous purines in the human body*, a view rendered especially plausible by the growing statistics on tissue enzymes. The inequalities in the quantitative aspects of this uric acid production are striking, a far larger proportion of uric acid being eliminated after ingestion of hypoxanthine and xanthine than after the amino-purines. We may call attention to the absence of any further metabolic disturbance associated with the introduction of the purines in the doses used. They did not act conspicuously as diuretics. The absence of any marked perversion of nitrogenous metabolism and the uniformity of the conditions observed is attested by the constancy of some of the urinary features, notably the endogenous creatinine elimination and the figures for ammonia-nitrogen and phosphorus.

Quite recently Plimmer, Dick, and Lieb<sup>1</sup> have advocated a different view regarding the origin of uric acid in the urine of man. They observed, in experiments on a single subject, that "administration of xanthine and guanine gave practically no rise in the uric acid output. Administration of very large quantities of purine in herring roe certainly increased the output of uric acid, but the excess of uric acid only corresponded to about one-tenth of the total purines ingested. Liebig's extract of meat caused a great increase in the excretion of uric acid, more than half the purine present in it being converted into uric acid." These investigators deny any significant relation between purines and uric acid in the subject. They reject the old view of Horbaczewski referring the increase in urinary uric acid to *destroyed* leucocytes, because in their subject increase in uric acid was not associated with decrease in the number of leucocytes. Plimmer, Dick, and Lieb advance the hypothesis that in leucocytes the end product of metabolism is uric acid; and they conclude that the uric acid of the urine is an expression of leucocyte metabolism. To account for the marked influence on uric acid output which is commonly found after ingestion of

<sup>1</sup> Plimmer, Dick, and Lieb: *Journ. of Physiol.*, xxxix, p. 98, 1909.

meat extract, they note that the latter causes leucocytosis and conclude that consequently an increase in the quantity of uric acid follows. It is suggested that the poisons of disease, *e.g.*, in gout, "call forth a larger number of leucocytes into the blood to act as scavengers and this is probably the reason why the Liebig's extract of meat cause such leucocytosis." These writers add that "further work will be necessary before the views here put forward can be regarded as established, but our results certainly throw considerable doubt on the prevalent opinion that the purines of the food and of the tissues are the sole source of the uric acid in the urine."

We cannot explain our own results on the basis of such an hypothesis. It will be noted in our protocols that an increased output of uric acid was attained just as well by feeding pure hypoxanthine as through the agency of the extract of meat with its hypothetical "toxins." One might assume that hypoxanthine itself induced the leucocytosis postulated by Plimmer's theory; but Krüger and Schmid,<sup>1</sup> who obtained a 62 per cent yield of uric acid from hypoxanthine and specially investigated this point, found no change in the leucocyte counts when hypoxanthine was fed. In Plimmer, Dick and Lieb's subject the increase in the output of uric acid nitrogen after feeding xanthine and guanine did not exceed 10 per cent of the purine nitrogen ingested. Our own experiments gave considerably higher figures, especially in the case of xanthine with which Krüger and Schmid also obtained rather slight increases (about 10 per cent). We cannot admit at present that the negative experiments quoted by others are adequate to disprove the current view that in man the food purines are directly related to the increased content of uric acid in the urine which follows their ingestion. On the other hand, our experience speaks against the probability of any *constant factor of destruction* related to the "integrative factor" of Burian and Schur. The variables are too numerous; for rate of absorption, concentration of the purines in the circulating media and of enzymes in the metabolic organs, as well as individuality of equipment, must play a variable part. To this may be added the recently suggested possibility of a concomitant resynthesis of uric acid from its decomposition products.<sup>2</sup>

<sup>1</sup> Krüger and Schmid: *Zeitschr. f. physiol. Chem.*, xxxiv, p. 556, 1902.

<sup>2</sup> Cf. Ascoli and Izar: *Zeitschr. f. physiol. Chem.*, lviii, p. 529, 1909; Bezzola, Izar, and Preti: *ibid.*, lxii, p. 229, 1909; Preti: *ibid.*, p. 354.



# THE STUDY OF ENZYMES BY MEANS OF THE SYNTHETICAL POLYPEPTIDS.

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Our knowledge of the proteolytic ferments has been obtained by a study of the action of the extracts and secretions of the organs of various plants and animals upon the proteids or their intermediary decomposition products. Until a few years ago substances of unknown constitution were used to detect the presence or absence of a proteolytic enzyme. However, the untiring efforts of Emil Fischer in the synthesis of the polypeptids have furnished us with substances of known constitution, which have been used to study the action of the proteolytic enzymes more closely.

About seven years ago E. Fischer and P. Bergell<sup>1</sup> tested the action of trypsin upon several dipeptids and derivatives of dipeptids. They found that some of these compounds were hydrolyzed to the amino-acids while others were not. Thus it was discovered that glycyl-l-tyrosin was changed into glycocoll and l-tyrosin by the action of pancreatin. A short time after this discovery E. Fischer and E. Abderhalden<sup>2</sup> published an account of the action of trypsin, obtained from a pancreatic fistula, upon twenty-nine different polypeptids. They found that fourteen of these were hydrolyzed while the remaining fifteen were not, using both the chemical and the optical methods to prove this point. Thus alanyl-glycin, the dipeptid which was used as the test object in this publication, was hydrolyzed by pancreatic juice. A subsequent article<sup>3</sup> showed the remarkable specificity of the proteolytic enzymes—namely, that

<sup>1</sup> Fischer and Bergell: *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 2592, 1903; xxxvii, p. 3104, 1904.

<sup>2</sup> Fischer and Abderhalden: *Zeitschr. f. physiol. Chem.*, xlvi, p. 52, 1905.

<sup>3</sup> Fischer and Abderhalden: *Zeitschr. f. physiol. Chem.*, li, p. 264, 1907.

only those polypeptids which contained the natural components in combination were hydrolyzed, while the ferment did not act upon those polypeptids which contained the unnatural amino-acids in combination with each other. Thus it was shown that of the four possible stereoisomeric alanyl-alanin dipeptids only one—d-alanyl-d-alanin—was hydrolyzed by trypsin.

Abderhalden and his co-workers have demonstrated the presence of these polypeptid-hydrolyzing enzymes in yeast, papajotin, in sprouted and unsprouted seeds, in the organs of the ox, dog, rabbit and mouse, in the intestinal juice obtained from dogs by a fistula, in tumors of various kinds, and in the blood of the ox, horse, sheep, dog and rabbit.

In the hydrolysis of the dipeptids, there is only one possible way in which they can be hydrolyzed—that is, there is only one bond which is broken open by the action of the ferment; for instance, alanyl-glycin can only be hydrolyzed in one way with the formation of alanin and glycocoll. However, in the case of the tripeptids the matter is not so simple and a closer study of the action of certain enzymes upon two tripeptids has brought forth a very interesting fact.<sup>1</sup>

A tripeptid containing l-leucin, glycin and d-alanin, namely, l-leucyl-glycyl-d-alanin, has been prepared synthetically by E. Fisher and J. Steingröver. The optical properties of this tripeptid, as well as of the possible substances formed by hydrolysis, are given in the following scheme:<sup>2</sup>

+ 52°		
1.	2.	3.
l-Leucyl	- glycyl	- d-alanin.
-13°	0°	+2°
+ 172°		
- 73°		

<sup>1</sup> Abderhalden and Koelker: *Zeitschr. f. physiol. Chem.*, liv, p. 363, 1908; lv, p. 425, 1908; Abderhalden and Brahm: *ibid.*, lvii, p. 346, 1908.

<sup>2</sup> The values given for the optical rotations are one hundredth of the molecular rotations.



If this tripeptid is subjected to the action of a proteolytic enzyme it can easily be seen that there are several ways in which the hydrolysis may be brought about; first, the enzyme may break the union between the first and second amino-acid radicals with the formation of l-leucin and glycyl-d-alanin. The optical rotation of the tripeptid is to the right; with the formation of the l-leucin and glycyl-d-alanin, the positive rotation of such a solution if observed in the polariscope would gradually decrease and finally become negative, and the negative rotation would increase until it reaches a maximum. In the second case the linking in the chain may be broken between the second and third amino-acid radicals with the formation of l-leucyl-glycin and d-alanin. The optical rotation of these two products is several times as great as the optical rotation of the tripeptid, as can be seen from the diagram. If therefore these two substances should be formed by the action of the ferment the positive rotation of the solution of the tripeptid would gradually increase in the positive direction until it would reach a maximum. In the third possibility, the linking in the chain may be broken simultaneously between the first and second and between the second and third amino-acid radicals, with the formation of leucin, glycocoll and d-alanin. The rotation of these three substances together would cause a slight laevo rotation. If therefore the enzyme should hydrolyze the tripeptid at these two linkings simultaneously, the optical rotation of such a solution of the tripeptid when observed in the polariscope would gradually sink from a positive rotation to a weak negative rotation and remain constant. The polariscope therefore will show us just how the proteolytic enzyme attacks the tripeptid. Two different enzymes were allowed to react with this tripeptid. It was found that trypsin first attacked the linking between the second and third amino-acid radical while the proteolytic enzyme of yeast attacked first the linking between the first and second amino-acid radical. The solution of this problem is a striking example of the value of the optical methods. Here it would be almost impossible to solve a problem of this kind without the use of the polariscope. The second tripeptid which was studied from the same point of view was glycyl-d-alanyl-glycin. The diagram below gives the optical

rotations of the tripeptid and the various possible hydrolytic products, in terms of 1/100 of the molecular rotations:

- 130°		
1.	2.	3.
Glycyl	- d-alanyl	- glycyl
0°	+ 2°	0°
- 73°		
+ 73°		

Here we have the three possibilities as in the case of l-leucyl-glycyl-d-alanin, and the diagram explains the changes which we can expect in the optical rotation of such a solution when it is acted upon by a proteolytic enzyme. The action of trypsin, erepsin and pressed yeast juice were studied upon this tripeptid, and it has been shown that all three of these enzymes first attacked this tripeptid at the same linking, namely, between the first and second amino-acid radical, with the formation of glycocoll and d-alanyl-glycin.

It is the object of this communication to show that the study of the proteolytic enzymes can be realized with one of the easily accessible dipeptids. The preparation of the optically active polypeptids is not only very laborious but also very expensive. It can easily be seen from the results presented in this article that racemic alanyl-glycin can be used with great accuracy for the study of the proteolytic enzymes using the optical method.

#### THE PRINCIPLES OF THE OPTICAL METHOD.

For the study of the polypeptolytic enzyme racemic alanyl-glycin has been chosen for two reasons: firstly, it is hydrolyzed by trypsin; and secondly, it is the most easily accessible dipeptid which has the necessary physical properties for the application of the optical method.

When racemic alanyl-glycin in a half-normal solution is subjected to the action of the enzyme in pressed yeast juice only the one component, the d-alanyl-glycin is hydrolyzed with the formation of d-alanin and glycocoll, while the other component, the l-alanyl-glycin remains unchanged. The solution before the action

the enzyme is entirely inactive toward polarized light. With the formation of the d-alanin, however, the optical rotation of both the d-alanin and the l-alanyl-glycin can easily be detected in the polariscope, and as the hydrolysis proceeds the rotation of the plane of polarized light increases until it reaches a maximum. In all the experiments in this publication, unless especially mentioned, the amount of the dipeptid used is always the same, namely, 1/400 mol. of the racemic compound, the concentration of the solution is also always the same, namely, one-half normal, and the angle of rotation is always observed in a one decimeter tube at 37°, using a Welsbach burner as the source of light. By adhering to exactly the same conditions throughout all the experiments, the change in the observed angle of rotation will be a measure for the amount of d-alanyl-glycin hydrolyzed.

It was important to compare this maximum rotation found to that which we could expect by a complete hydrolysis. First, however, it was necessary to determine the specific rotation of l-alanyl-glycin at various concentrations. The specific rotation of d-alanyl-glycin,<sup>1</sup> the optical antipode, has been found to be + 50.3° (±0.1°) in a 10 per cent aqueous solution. Some l-alanyl-glycin was prepared synthetically and its specific rotations determined in various concentrations. The data are given in the following table:—

CONCENTRATION OF THE SOLUTION IN PER CENT	SPECIFIC GRAVITY $d_4^{20}$	CALCULATED SPECIFIC ROTATION $[\alpha]_D^{20}$
9.955	1.035	— 50.0° (± 0.2°)
7.983	1.028	— 50.6° (± 0.2°)
4.981	1.017	— 51.6° (± 0.2°)
3.99	1.013	— 51.6° (± 0.2°)
2.49	1.008	— 52.2° (± 0.4°)
1.25	1.003	— 52.6° (± 0.8°)
0.62	1.001	— 51.9° (± 1.7°)
0.31	1.000	— 51.° (± 3.4°)
0.16	1.000	— 49.° (± 7.0°)

This table shows that the specific rotation of l-alanyl-glycin increases slightly with dilution. The concentration of l-alanyl-

<sup>1</sup> Fischer: *Ber. d. deutsch. chem. Gesellsch.*, li, p. 852, 1908.

glycin in a half-normal solution of the racemic dipeptid is 3.56 per cent.<sup>1</sup> Its specific rotation in this concentration is  $-51.6$  ( $\pm 0.2$ ) (see table). The specific rotation of d-alanin is  $+2.7^\circ$  ( $\pm 0.1$ ). In the following scheme the rotations of the substances formed by complete asymmetrical hydrolysis of a half-normal solution of racemic alanyl-glycin are represented, measured in a one decimeter tube at  $20^\circ$  with sodium light.

$$\begin{array}{ccc}
 & +0.06^\circ & 0^\circ \\
 & \underbrace{\hspace{1.5cm}} & \underbrace{\hspace{1.5cm}} \\
 0^\circ \left\{ \begin{array}{l} \text{d-alanyl - glycin} \\ \text{l-alanyl - glycin} \end{array} \right. & & \\
 & \underbrace{\hspace{1.5cm}} & \\
 & -1.89^\circ & 
 \end{array}$$

We can expect, therefore, a laevo rotation of  $-1.83^\circ$ . However, the rotation after the action of the enzyme has been determined in a one decimeter tube at  $20^\circ$  with sodium light and found to be  $-1.70^\circ$ . These two values do not correspond absolutely, but they are indeed close. In fact, so close that they justify the application of this method to the study of the proteolytic enzymes. The accuracy with which this optical method can be used is far beyond the accuracy of the chemical method. Besides this, it has the advantage over the latter from the fact that it requires much less of the dipeptid and can be performed in a much shorter time.

To be absolutely certain that l-alanyl-glycin, d-alanin and glycocoll were formed by the action of pressed yeast juice upon the racemic dipeptide, the following experiment was performed:—48.5 cc. of a normal solution of racemic alanyl-glycin (7.08 grams of the dipeptid) were mixed with 29.1 cc. of water and 19.4 cc. of pressed yeast juice. The solution was allowed to digest at  $37^\circ$  until its optical rotation had reached  $-1.51^\circ$ , measured in a 1 dm. tube at  $37^\circ$  with white light. The solution was then boiled to destroy the enzyme and the three products formed isolated by the regular method.<sup>2</sup> The yields of the raw products were as follows: 1.47 gm. of glycine ester hydrochloride; 2.00 gm. of the distilled amino-acid

<sup>1</sup> The specific gravity of the solution of the dipeptid was determined before and after the hydrolysis by the enzyme and found to be 1.027 and 1.0 respectively.

<sup>2</sup> Fischer and Abderhalden: *Zeitschr. f. physiol. Chem.*, xlv, p. 52, 1901.

which consist mainly of d-alanin, and 3.00 gm. of l-alanyl-glycin-anhydride.

For analysis the glycine ester hydrochloride was recrystallized once from hot alcohol.

Melting point =  $144^{\circ}$  (uncorrected).

0.1977 gm. substance required 14.3 cc. of  $\frac{N}{10}$  silver nitrate solution (Volhard).

	Calculated for $C_4H_9O_2N.HCl$ : Per Cent	Found: Per Cent
Cl.....	25.4	25.6

The d-alanin was recrystallized once from hot water. The optical rotation of its hydrochloride was found to be  $+9.3^{\circ}$  ( $\pm 0.2^{\circ}$ ).

#### ANALYSIS:

0.2039 gm. substance gave 0.2999 gm.  $CO_2$ , and 0.1450 gm.  $H_2O$ .

	Calculated for $C_3H_7O_2N$ : Per Cent	Found: Per Cent
C.....	40.4	40.1
H.....	7.92	7.96

The l-alanyl-glycin anhydride was recrystallized once from hot alcohol. The specific rotation in water was found to be  $+3.1^{\circ}$  ( $\pm 0.4^{\circ}$ ).

#### ANALYSIS:

0.1602 gm. substance gave 0.2755 gm.  $CO_2$  and 0.0916 gm.  $H_2O$ .

	Calculated for $C_5H_8O_2N_2$ : Per Cent	Found: Per Cent
C.....	46.88	46.90
H.....	6.25	6.40

The chemical composition of the three substances corresponds to the theory. The value of the specific rotations is a trifle smaller than the optically pure substances. But this can be expected, for in the process of isolation a small amount of the substances is racemicized. The isolation of these three substances proves conclusively that the hydrolysis has been brought about by the enzyme with the formation of d-alanin, glycine and l-alanyl-glycine.

#### THE ACCURACY OF THE OPTICAL METHOD.

In the estimation of the activity of one enzyme solution as compared to that of another enzyme solution, the size of the change in

rotation in a given time will give us an idea as to the comparative hydrolytic power of the two unknown ferment solutions. In all the experiments with alanyl-glycin (unless special mention is made) the amount of the racemic dipeptid used and the concentration of the solution are always the same—namely, 1/400 mol. of the dipeptid (0.365 gm.) and one-half normal solution. The ferment, therefore, has exactly the same amount of the dipeptid to decompose in every experiment; if more of the active principle is present more of the dipeptid will be changed in a given time, and consequently the change in the rotation in that time must be greater than when less of the active principle is taken. This has been shown very accurately in some experiments with d-alanyl-d-alanin.<sup>1</sup> Using alanyl-glycin, it has been shown above in the principles of the method that the complete hydrolysis is designated by a rotation of  $-1.51^\circ$  when measured in a 1 dm. tube at  $37^\circ$  with white light. To test the accuracy of the method, two series of experiments have been made in which equal volumes of the enzyme solution have been added to the solution of the dipeptid and the changes in rotation after equal time periods determined in the polariscope under the regular conditions mentioned above.

## SERIES I.

**A**

1.00 cc. of pressed yeast juice which had been standing at a temperature of  $15^\circ$ – $20^\circ$  for 6 weeks.

1.50 cc. of distilled water.

2.50 cc. of normal racemic alanyl-glycin. (1/800 mol. d-alanyl-glycin).

**B**

1.00 cc. of the same pressed yeast juice as in *a*.

1.50 cc. of distilled water.

2.50 cc. of the same solution of dipeptid as in *A* (1/800 mol. d-alanyl-glycin).

TIME	CORRECTED ANGLE OF ROTATION	TIME	CORRECTED ANGLE OF ROTATION
Minutes	Degrees	Minutes	Degrees
0.....	0.00	0.....	0.00
45.....	–0.07	45.....	–0.07
150.....	–0.24	150.....	–0.23
275.....	–0.40	275.....	–0.43
395.....	–0.60	395.....	–0.59
545.....	–0.76	545.....	–0.77
650.....	–0.87	650.....	–0.89
Hours		Hours	
28.....	–1.47	28.....	–1.48

<sup>1</sup> Abderhalden and Koelker: *Zeitschr. f. physiol. Chem.*, li, p. 309, 1907.

## SERIES II.

## A

1.12 cc. of pressed yeast juice, the same as in Series I, but after it had been dialyzed in parchment paper for two days.

1.38 cc. of water.

2.50 cc. of a normal solution of racemic alanyl-glycin (1/800 mol. d-alanyl-glycin).

## B

1.12 cc. of the same pressed yeast juice as in A.

1.38 cc. of water.

2.50 cc. of the same normal alanyl-glycin solution as in A (1/800 mol. d-alanyl-glycin).

TIME Minutes	CORRECTED ANGLE OF ROTATION Degrees	TIME Minutes	CORRECTED ANGLE OF ROTATION Degrees
0.....	0.00	0.....	0.00
45.....	-0.10	45.....	-0.08
150.....	-0.36	150.....	-0.33
275.....	-0.59	275.....	-0.59
395.....	-0.82	395.....	-0.79
545.....	-0.99	545.....	-0.99
650.....	-1.14	650.....	-1.10
Hours		Hours	
28.....	-1.49	28.....	-1.48

These two series suffice to show the accuracy of this optical method, the errors in the observed angles of rotation being within the error of the instrument—namely,  $\pm 0.02^\circ$ . With the aid of such an accurate method, we can expect to detect very fine differences in the hydrolytic power of two enzyme solutions, and thus make it possible to study the influence of various factors, not only upon the enzyme during the time of hydrolysis, but also upon the production of the ferment.

## QUESTION OF BACTERIAL ACTION.

In all the experiments the normal solutions of the polypeptids, the solutions of the enzyme and the solutions during the time of the observations of the hydrolysis are continually covered with toluol to preserve them.

In order to decide definitely whether or not such bacteria could develop which would cause the hydrolysis of the polypeptids, the following experiments were performed:—

A one-half normal solution of d-alanyl-d-alanin was allowed stand at a temperature of  $15^{\circ}$ - $20^{\circ}$  for a period of 13 months. The optical rotation of this solution in a 1 dm. tube at  $35^{\circ}$  with sodium light was determined beforehand and found to be  $-1.57^{\circ}$  ( $\pm 0.2^{\circ}$ ). At the end of the 13 months the angle of rotation was again determined under exactly the same conditions and found to be  $-1.6$  ( $\pm 0.2^{\circ}$ ). These two observations are just within the limit of error of the instrument; the experiment shows that after standing for 13 months the dipeptid has not been altered at all, either by the action of bacteria or by the action of the water in which it was dissolved. The solution was now allowed to stand in a thermostat at  $37^{\circ}$  for a period of ten days and the rotation of the solution was again taken, and found to be  $-1.64^{\circ}$ . Here there is a very slight increase in rotation. Had any hydrolysis of the dipeptid taken place the rotation of the solution necessarily would have decreased (This slight increase was caused by a slight evaporation, as the solution was allowed to remain in the polariscope tube for the ten days). Bacteria could not have developed during the time of observation. A similar experiment was made with racemic alanyl-glycin. A normal solution of this dipeptid was also allowed to stand for a period of 13 months at a temperature of from  $15^{\circ}$  to  $20^{\circ}$ . The solution was inactive to polarized light both before and after. In order to test if the dipeptid had been changed or not the solution was subjected to the action of pressed yeast juice at  $38^{\circ}$ , and it was found that it behaved exactly as a fresh solution of racemic alanyl-glycin, as the following experiment shows:

## I.

2.50 ccm. of a normal solution of racemic alanyl-glycin which had been standing 13 months (1/800 mol. d-alanyl-glycin).

1.50 ccm. of physiological saline solution.

1.00 ccm. of pressed yeast juice.

## II.

2.50 ccm. of a freshly prepared normal solution of racemic alanyl-glycin (1/800 mol. d-alanyl-glycin).

1.50 ccm. of physiological saline solution.

1.00 ccm. of pressed yeast juice

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<sup>1</sup> Abderhalden and Koelker: *Zeitschr. f. physiol. Chem.*, li, p. 309, 1906



TIME	CORRECTED ANGLE	TIME	CORRECTED ANGLE
Minutes	OF ROTATION <sup>1</sup>	Minutes	OF ROTATION
	Degrees		Degrees
0.....	0.00	0.....	0.00
20.....	-0.54	20.....	-0.51
34.....	-0.75	34.....	-0.72
61.....	-1.01	61.....	-1.04
88.....	-1.18	88.....	-1.19
122.....	-1.32	122.....	-1.32
153.....	-1.40	153.....	-1.40
203.....	-1.47	203.....	-1.46
273.....	-1.48	273.....	-1.48
453.....	-1.51	453.....	-1.50

The changes in rotation in I and II within the same time periods are exactly the same, showing that exactly the same amount of racemic alanyl-glycin is present in each case. The experiments show conclusively that the dipeptids have not been affected in any way, either by the action of bacteria or by the action of water, after standing for a period of 13 months.

#### PREPARATION OF THE ENZYME SOLUTION.

Thus far no experiments have been made upon the polypeptids to test the hydrolytic power of extracts obtained by auto-digestion of yeast cells as compared to the pressed yeast juice obtained by Buchner's process.

Three different extracts were made:

*Yeast Extract No. 1.* 30 grams of Fleischman's compressed yeast were mixed with 30 cc. of physiological saline solution and digested at 38° for 48 hours, using toluol as preservative. At the end of this period the solution was filtered and its hydrolytic power tested.

*Yeast Extract No. 2.* 30 grams of the same sample of Fleischman's yeast as in No. 1 were dried in a vacuum desiccator. The dried material was pulverized with a little sand and then digested with 30 cc. of physiological saline solution for 48 hours at 38° after the addition of toluol. Then filtered and its hydrolytic power tested.

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<sup>1</sup> At times the solution of the enzyme itself turns the plane of polarized light slightly—in the concentration above however (1.0 cc. of the ferment solution to 4.0 cc. of water) it is only in rare instances more than  $\pm 0.04^\circ$  in a 1 dm. tube. A correction of the observed angle is necessary for every different ferment solution. The solution of the enzyme itself is always subjected to self digestion at 38° until its rotation becomes constant, requiring from 20 to 50 hours.

*Yeast Extract No. 3.* 30 grams of the same sample of yeast as in 1 and 2 were treated with 5 parts of absolute alcohol and allowed to remain in contact with the liquid for 15 minutes, mixing well in the meantime. The alcohol was then poured off and the cells treated with the same amount of alcohol twice for the same time. The yeast cells were then filtered off and washed with ether and dried in a vacuum. The light powder obtained was digested with 30 cc. of physiological salt solution at 38° for 48 hours after the addition of toluol. The material was filtered and its strength tested.

In the following series the same volume of the enzyme solution has been taken in each experiment:

YEAST EXTRACT NO. I.		YEAST EXTRACT NO. II.		YEAST EXTRACT NO. III.		BUCHNER'S PRESSE- YEAST JUICE, PREPARED FROM FLEISCHMAN'S COMPRESSED YEAST —	
2.50 cc. of a normal solution of alanyl-glycin (1/800 mol. d-alanyl-glycin).		2.50 cc. of a normal solution of racemic alanyl-glycin as in No. I (1/800 mol. d-alanyl-glycin).		2.50 cc. of the same alanyl-glycin solution as in I (1/800 mol. d-alanyl-glycin).		2.50 cc. of the same alanyl-glycine solution as in I	
1.50 cc. of physiol. salt solution.		1.50 cc. of physiol. salt solution.		1.50 cc. of physiol. salt solution.		1.50 cc. of physiol. salt solution.	
1.00 cc. of yeast extract No. I.		1.00 cc. of yeast extract No. II.		1.00 cc. of yeast extract No. III.		1.00 of pressed yeast juice.	
TIME	CORRECTED ANGLE OF ROTATION	TIME	CORRECTED ANGLE OF ROTATION	TIME	CORRECTED ANGLE OF ROTATION	TIME	CORRECTED ANGLE OF ROTATION
<i>Hours</i>	<i>Degrees</i>	<i>Hours</i>	<i>Degrees</i>	<i>Hours</i>	<i>Degrees</i>	<i>Minutes</i>	<i>Degrees</i>
0	0.00	0	0.00	0	0.00	0	0.00
24	−0.09	21	−0.32	21	−0.95	20	−0.51
48	−0.13	75	−0.66	46	−1.30	34	−0.72
72	−0.18	93	−0.80	75	−1.34	61	−1.04
<i>Days</i>				93	−1.36	88	−1.19
17	−0.71					122	−1.32
						153	−1.40
						273	−1.48
						453	−1.50

The experiment shows that the best extract is obtained if the cells are dried with alcohol and ether. But the hydrolytic power of such an extract does not compare with that solution of enzyme

which is obtained by Buchner's grinding and pressing process. For the study of this enzyme it is therefore best, at least for the present, to use that material which is prepared by Buchner's method.

#### EFFECT OF PRECIPITATION OF PRESSED YEAST JUICE WITH ALCOHOL.

An attempt was made to precipitate the active dipeptid-hydrolyzing principle of pressed yeast juice with absolute alcohol. 10 cc. of the juice were poured into 40 cc. of absolute alcohol and allowed to stand for five minutes. The precipitate was then filtered off and washed with absolute alcohol and immediately dried *in vacuo*. The precipitate weighed 0.505 gram. It was dissolved in 10 cc. of physiological saline solution, filtered and its hydrolyzing power tested below (No II). The alcoholic filtrate was immediately evaporated *in vacuo* at 35° to a pasty mass and dissolved in 10 cc. of physiological saline solution and filtered. The following series of experiments shows the hydrolyzing power of the juice before and after precipitation:

I THE UNPRECIPITATED JUICE		II THE ALCOHOLIC PRECIPITATE		III THE ALCOHOLIC FILTRATE	
1.00 cc. of the juice before precipitation.		2.00 cc. of the redissolved alcoholic precipitate.		2.00 cc. of the alcoholic filtrate after being evaporated and redissolved.	
1.50 cc. of physiol. saline solution.		0.50 cc. of physiol. saline solution.		0.50 cc. of physiol. saline solution.	
2.50 cc. of normal d-l-alanyl-glycin solution.		2.50 cc. of normal d-l-alanyl-glycin solution.		2.50 cc. of normal d-l-alanyl-glycin solution.	
TIME	CORRECTED ANGLE OF ROTATION	TIME	CORRECTED ANGLE OF ROTATION	TIME	CORRECTED ANGLE OF ROTATION
<i>Minutes</i>	<i>Degrees</i>	<i>Hours</i>	<i>Degrees</i>	<i>Hours</i>	<i>Degrees</i>
0	0.00	0	0.00	0	0.00
20	-0.51	1	-0.17	1	0.00
34	-0.72	3	-0.30	3	0.00
61	-1.04	4½	-0.66	4½	0.00
88	-1.19	16	-1.28	16	+0.01
122	-1.32	19	-1.37	39	+0.02
153	-1.40	24	-1.43		
203	-1.46	39	-1.43		
453	-1.50				

On comparison of the optical rotations it can easily be seen that the alcoholic precipitation has injured the active principle to a very great degree. Whereas the original juice hydrolyzed about one-half of the dipeptid within 34 minutes, the material which was precipitated by alcohol required over 4½ hours to hydrolyze the same amount of dipeptid, while the alcoholic filtrate contained no active principle whatever. Thinking that the alcoholic filtrate might contain some substance which had the property of accelerating the action of the dissolved precipitate, the following experiment was performed in which both the dissolved precipitate with the solution of the evaporated alcoholic filtrate were allowed to act upon the dipeptid:

2.00 cc. of the dissolved precipitate  
 2.50 cc. of the dissolved alcoholic filtrate.  
 2.50 cc. of the normal d-l-alanyl-glycin solution.

TIME	CORRECTED ANGLE OF ROTATION
<i>Hours</i>	<i>Degrees</i>
0	0.00
1½	0.15
3	0.29
6	0.53
23	1.20

The result, however, shows that there was no accelerating substance in the alcoholic filtrate. In fact, the addition of the same seemed to inhibit the rate of hydrolysis. This one experiment shows that the alcoholic precipitation cannot be used to isolate the active principle from the other material present in pressed yeast juice. Consequently the effect of dialysis has been studied to see if this method could be used to any advantage.

#### EFFECT OF DIALYSIS UPON THE ENZYME SOLUTION.

The influence of dialysis has been studied upon three different solutions of pressed yeast juice (P. Y. J.) prepared from the same sample of yeast (Fleischman's compressed yeast), which are designated as P. Y. J. III<sup>b</sup>, III<sup>c</sup>, and III<sup>e</sup>, and upon a fourth solution prepared from Corby's compressed yeast, designated as P. Y. J. V. All the material was prepared by Buchner's grinding and pressing method.

A. P. Y. J. III<sup>b</sup>. 10 cc. of this juice were subjected to dialysis with distilled water in one of the diffusion tubes No. 579 prepared by Schleicher and Schüll. The water was changed every four hours during the day and the tube allowed to remain in 60 cc. of water over night. The time of the dialysis was 44 hours; the amount of water at each change 60 cc.; the volume of the solution afterwards was 11.3 cc. The solids in solution beforehand, dried at 105°, were 11.1 per cent; after being subjected to dialysis, the solution contained 1.01 per cent solids, about 90 per cent having been removed by this procedure. The activities of the two solutions are expressed in the following series:

I		II	
P. Y. J. III <sup>b</sup> . 2.50 cc. of the normal solution of rac. alanyl-glycin. 1.50 cc. of water. 1.00 cc. of P. Y. J. III <sup>b</sup> .		P. Y. J. III <sup>b</sup> subjected to di- alysis for 44 hours. 2.50 cc. of the dipeptid solution. 1.50 cc. of water. 1.00 cc. of P. Y. J. III <sup>b</sup> subjected to dialysis.	
TIME Minutes	CORRECTED ANGLE Degrees	TIME Minutes	CORRECTED ANGLE Degrees
0.....	-0.00	0.....	-0.00
40.....	-0.14	40.....	-0.16
144.....	-0.52	144.....	-0.62
270.....	-0.84	270.....	-1.05
385.....	-1.05	385.....	-1.29
470.....	-1.19	470.....	-1.37
Days		Days	
3.....	-1.50	3.....	-1.49

The experiment shows that the activity of the enzyme was greater in the solution which had been subjected to dialysis. The acidity of the original solution was rather marked, and thinking that this could account for the lower rate of hydrolysis, the following experiment was performed:

2.00 cc. of the original P. Y. J. III<sup>b</sup>, were neutralized (litmus) with a 1 per cent soda solution. 0.50 cc. of this solution was required. 1.25 cc. were tested in comparison to 1.00 cc. of the original solution.

I  
P. Y. J. III<sup>b</sup>.  
2.50 cc. of the dipeptid solution.  
1.50 cc. of water.  
1.00 cc. of P. Y. J. III<sup>b</sup>.

II  
P. Y. J. III<sup>b</sup> neutralized.  
2.50 cc. of the dipeptid solution  
1.25 cc. of water.  
1.25 cc. of the neutralized P. Y. J. III<sup>b</sup>.

TIME	CORRECTED ANGLE	TIME	CORRECTED ANGLE
Minutes	OF ROTATION	Minutes	OF ROTATION
	Degrees		Degrees
0.....	0.00	0.....	0.00
40.....	-0.17	40.....	-0.26
120.....	-0.43	120.....	-0.66
190.....	-0.62	190.....	-0.93
255.....	-0.75	255.....	-1.11
325.....	-0.90	325.....	-1.29
410.....	-1.04	410.....	-1.42
Hours		Hours	
24.....	-1.50	24.....	-1.48

This last experiment shows that the acid present inhibits the rate of hydrolysis to a marked degree and probably explains the effect of dialysis which naturally would remove most of the acid present. In an earlier paper it has been shown that very small amounts of hydrochloric acid can prevent entirely the hydrolysis of alanyl-alanin by this same enzyme.<sup>1</sup>

B. P. Y. J. III<sup>d</sup>. Three different solutions of this juice of 10.0 cc. each were subjected to dialysis in three of the parchment tubes prepared by Schleicher and Schüll for a period of six days. The total amount of water used in each of the three experiments was 700 cc. The volume after dialysis in all three tubes was 50.0 cc., so that 1.00 cc. of the original solution of the enzyme was equivalent to 1.66 cc. of the solution which had been subjected to dialysis. The original solution contained 9.31 per cent solids, The solution after dialysis contained 0.45 per cent.

The following series shows the comparative hydrolytic power of the two solutions:

I  
P.Y. J. III<sup>d</sup>.  
2.50 cc. of normal racemic alanyl-glycin solution.  
1.50 cc. water.  
1.00 cc. P. Y. J. III<sup>d</sup>.

II  
P. Y. J. III<sup>d</sup>. subjected to dialysis for 6 days.  
2.50 cc. of normal racemic alanyl-glycin solution.  
0.84 cc. water.  
1.66 cc. P. Y. J. III<sup>d</sup>. subjected to 6 days dialysis.

<sup>1</sup> Abderhalden and Koelker: *Zeitschr. f. physiol. Chem.*, liv, p. 384, 1908;

TIME	CORRECTED ANGLE	TIME	CORRECTED ANGLE
Minutes	OF ROTATION	Minutes	OF ROTATION
	Degrees		Degrees
0.....	0.00	0.....	0.00
32.....	-0.10	32.....	0.12
100.....	-0.39	100.....	-0.35
185.....	-0.72	185.....	-0.55
295.....	-1.03	295.....	-0.80
405.....	-1.23	405.....	-0.97
525.....	-1.39	525.....	-1.14
Hours		Hours	
25.....	-1.49	25.....	-1.47

The solution of the enzyme after 6 days' dialysis has now lost in its power to hydrolyze the dipeptid. It seems therefore, that a short dialysis yields a more active ferment, and by prolonging the dialysis the activity of the material in the tube is lessened. In the next series with P. Y. J. III<sup>e</sup>, this is shown definitely to be the case with this special material. The loss of the active principle by prolonged dialysis suggested the possibility that part of the active principle passed through the parchment membrane. Consequently the entire dialysate, 2100 cc., was evaporated *in vacuo* at 37° almost to dryness and enough water added to make the volume up to 30 cc. and filtered; 1.00 cc. of this filtrate was mixed with 2.50 cc. of the solution of dipeptid and 1.50 cc. of water. This solution was allowed to digest at 37° for 10 days. The rotation under the regular conditions was 0° before digestion and -1.44° afterward. The experiment shows that the dialysate contained the active principle, proving that *the active principle which causes the hydrolysis of alanyl-glycin has the property of dialyzing through parchment.*

This one experiment was so encouraging that the effect of dialysis was studied more closely upon P. Y. J. V. (see below). The following series shows the influence of shortened and prolonged dialysis upon P. Y. J. III<sup>e</sup>:

C. P. Y. J. III<sup>e</sup>. The activity of the enzyme solution was tested after two, four, and seven days dialysis. The method of dialysis was the same as in the preceding experiment. It was necessary in each case to determine the volume of the fluid after dialysis to find its equivalent of the original solution.

SERIES A.

P. Y. J. III<sup>e</sup>  
2.50 cc. of the solution of the dipeptid.  
1.50 cc. of water.  
1.00 cc. of P. Y. J. III<sup>e</sup>

P. Y. J. III<sup>e</sup> subjected to dialy sis for 2 days.  
2.50 cc. of the solution of th dipeptid.  
1.38 cc. of water.  
1.12 cc. of P. Y. J. III<sup>e</sup> subjecte to 2 days dialysis.

TIME	CORRECTED ANGLE	TIME	CORRECTED ANGLE
Minutes	Degrees	Minutes	Degrees
0.....	0.00	0.....	0.00
45.....	0.07	45.....	−0.10
150.....	−0.24	150.....	−0.36
275.....	−0.40	275.....	−0.59
395.....	−0.60	395.....	−0.82
545.....	−0.76	545.....	−0.99
650.....	−0.87	650.....	−1.14
Days		Days	
5.....	−1.47	5.....	−1.49

SERIES B

Performed 5 Days later than Series A.

P. Y. J. III <sup>e</sup> 2.50 cc. of the dipeptid to solution. 1.50 cc. of water. 1.00cc. of P. Y. J. III <sup>e</sup> .		P. Y. J. III <sup>e</sup> subjected to 4 days dialysis. 2.50 cc. of the dipeptid solution 1.40 cc. of water. 1.10 cc. of P. Y. J. III <sup>e</sup> subjected to 4 days dialysis.		P. Y. J. III <sup>e</sup> subjected to 7 days dialysis (in this case running water was used outside the tube). 2.50 cc. of the dipeptid solution. 1.64 cc. of water. 0.86 cc. of the enzyme solution.	
TIME	CORRECTED ANGLE	TIME	CORRECTED ANGLE	TIME	CORRECTED ANGLE
Minutes	Degrees	Minutes	Degrees	Minutes	Degrees
0	−0.00	0	0.00	0	0 00
200	−0.31	200	−0.23	200	−0.05
565	−0.80	565	−0.59	565	−0.08
Hours		Hours		Hours	
19½	−1.28	19½	−1.06	19½	−0.14
28½	−1.42	28½	−1.27	28½	−0.19
46	−1.50	46	−1.49	46	−0.27
Days		Days		Days	
11	.....	11	.....	11	−0.54



These two series show that short dialysis of this particular solution of enzyme has the effect of forming a more active enzyme, while by prolonged dialysis the active principle disappears; it passes through the parchment membrane as the next experiment shows conclusively.

D. P. Y. J. V. Three independent series of experiments were made in which the presence of the active principle was demonstrated in the dialysate and the gradual loss within the tubes shown.

Three independent experiments were made, in each of which 5.00 cc. of P. Y. J. V. were subjected to dialysis in the same kind of parchment fingers as used in the previous experiments. The amount of water used at each change was 35 cc. The various dialysates obtained from the first, second and third finger are given in the table below as Dialysate I, II and III, respectively. The table also gives the number of hours that the tube remained in contact with the dialysate. After each change a knife-point of calcium carbonate was added to the dialysate to prevent the destruction of the enzyme by any acid that could be present, and the fluid evaporated *in vacuo* to dryness. Each dialysate was tested separately for the presence of the ferment. Each residue obtained was digested with 2.50 cc. of physiological salt solution for a short time then 2.50 cc. of a one-half normal solution of racemic alanyl-glycin added and the material filtered. The amount of dipeptid used in this special series is just one-half the regular

TIME OF DIALYSIS	DIALYSATE NO. I		DIALYSATE NO. II		DIALYSATE NO. III	
	Rotation Before	Rotation After 14 Days Digestion	Rotation Before	Rotation After 14 Days Digestion	Rotation Before	Rotation After 14 Days Digestion
<i>Hours</i>	<i>Degrees</i>	<i>Degrees</i>	<i>Degrees</i>	<i>Degrees</i>	<i>Degrees</i>	<i>Degrees</i>
18	0.00	-0.46	0.00	-0.75	0.00	-0.67
28	0.00	-0.66	0.00	-0.70	0.00	-0.78
22	0.00	-0.71	0.00	-0.75	0.00	-0.23
26	0.00	-0.19	0.00	-0.46	0.00	-0.72
42			0.00	-0.40	0.00	-0.50
51			0.00	-0.55	0.00	-0.19

amount. Consequently a rotation of  $-0.75^{\circ}$  will designate a complete hydrolysis of the dipeptid present. The optical rotations of the various solutions obtained were determined immediately, and then each solution digested at  $37^{\circ}$  for 14 days. The optical rotations after this time were again determined. The table contains the rotations before and after digestion.

It is plainly seen that the dialysates in each case contained the dipeptid-hydrolyzing principle. This proves, without any doubt, that the enzyme has the property of passing through the parchment paper. On the other hand the gradual decrease of the activity of the principle within the tubes is demonstrated in the series given below.

SERIES A

P. Y. J. V  
2.50 cc. of the normal solution of  
rac. alanyl-glycin.  
1.50 cc. of physiol. salt solution.  
1.00 cc. of the enzyme.

P. Y. J. V subjected to 18 hours  
dialysis  
2.50 cc. of the dipeptid solution.  
1.50 cc. of physiol. NaCl.  
1.00 cc. of the enzyme solution.

TIME Minutes	CORRECTED ANGLE Degrees	TIME Minutes	CORRECTED ANGLE Degrees
0.....	0.00	0.....	0.00
39.....	-0.20	39.....	-0.10
62.....	-0.31	62.....	-0.19
87.....	-0.46	87.....	-0.31
145.....	-0.77	145.....	-0.56
209.....	-0.98	209.....	-0.81
285.....	-1.21	285.....	-1.08
363.....	-1.33	363.....	-1.28
Hours		Hours	
22.....	-1.53	22.....	-1.52

SERIES B

Three Days Later Than Series A.

P. Y. J. V  
2.50 cc. dipeptid solution.  
1.50 cc. physiol. NaCl.  
1.00 cc. of the enzyme solution.

P. Y. J. V subject to 4 days  
dialysis.  
2.50 cc. dipeptid solution.  
1.50 cc. physiol. NaCl.  
1.00 cc. of the enzyme solution.

TIME Minutes	CORRECTED ANGLE Degrees	TIME Minutes	CORRECTED ANGLE Degrees
0.....	0.00	0.....	0.00
90.....	-0.23	90.....	-0.17
170.....	-0.39	170.....	-0.33
240.....	-0.62	240.....	-0.47
385.....	-0.92	385.....	-0.71
470.....	-1.02	470.....	-0.82
600.....	-1.22	600.....	-1.03
Hours		Hours	
46.....	-1.48	46.....	-1.54

## SERIES C.

*15 Days Later Than Series B.*

P. Y. J. V

2.50 cc. of the dipeptid solution.

1.50 cc. of physiol. NaCl.

1.00 cc. of the enzyme solution.

P. Y. J. V subject to 8 days  
dialysis.

2.50 cc. of the dipeptid solution

1.90 cc. of physiol. NaCl.

0.60 cc. of the enzyme solution.

TIME Minutes	CORRECTED ANGLE Degrees	TIME Minutes	CORRECTED ANGLE Degrees
0.....	0.00	0.....	0.00
110.....	-0.17	110.....	-0.17
245.....	-0.41	245.....	-0.36
415.....	-0.70	415.....	-0.57
Hours		Hours	
24.....	-1.48	24.....	-1.42

This series of experiments shows that prolonged dialysis has the effect of removing the enzyme. It seems that some solutions of this enzyme are destroyed more rapidly than others. In each case it is necessary to make control tests, for at times it has been noticed that after standing at 15° for 24 hours there is a marked loss in activity. This is, however, more noticeable in solutions of this enzyme which have not been subjected to dialysis. Upon standing at 15° the solution which has been subjected to dialysis does not lose its activity so rapidly. The neutralization of the acid present, it seems to me, should be carried out in every solution of the enzyme tested and experiments are now in progress to find out how this can best be done to have as uniform a condition as possible. In some cases the amount of acid present can inhibit the hydrolysis completely, as the following experiment shows:

A specimen of Corby's compressed yeast which had been standing in an ice box at  $+4^{\circ}$  for 10 days was used to prepare some pressed yeast juice and then its power to hydrolyze alanyl-glycine was tested as given below in I. This juice had a marked acidic reaction and a portion of it was neutralized with a 1 per cent soda solution (2.00 cc. required 1.76 cc. of the soda solution, denoting a marked acidity. Litmus was used as indicator). The presence of the ferment in this neutralized solution is demonstrated in II.

## I.

P. Y. J. V from old yeast.  
2.50 cc. of the dipeptid solution.  
1.50 cc. of water.  
1.00 cc. of the enzyme solution.

## II.

P. Y. J. V same as in I but neutralized with soda.  
2.50 cc. of the dipeptid solution.  
0.62 cc. of water.  
1.88 cc. of the enzyme solution.

TIME Minutes	CORRECTED ANGLE Degrees	TIME Minutes	CORRECTED ANGLE Degrees
0.....	0.00	0.....	0.00
Days			
11.....	-0.05	11.....	-0.55

In I, we never could assume the presence of the enzyme, while in II, in which the same material tested in I had been neutralized, the experiment demonstrates positively the presence of the ferment. Although this experiment was performed with a sample of old yeast, it nevertheless demonstrates the necessity of testing for the enzyme in a neutral fluid.

## EFFECT OF EVAPORATION TO DRYNESS.

A. 2.00 cc. of pressed yeast juice, which had been subjected to dialysis in parchment for six days, were evaporated *in vacuo* at a temperature of  $15^{\circ}$  to dryness. The residue dissolved completely in 2.00 cc. of water, forming a perfectly clear solution. 1.66 cc. of this solution were added to 2.50 cc. of a normal solution of racemic alanyl-glycine and 0.84 cc. of water and its hydrolytic power tested as given below.

B. 1.66 cc. of the same solution of enzyme used in A, but which was not evaporated, were mixed with 2.50 cc. of a normal racemic alanyl-glycine solution and 0.84 cc. of water. The hydrolytic power of this solution was tested as shown below.

A		B	
Enzyme Solution Evaporated.		Enzyme Solution Not Evaporated.	
TIME	CORRECTED ANGLE OF ROTATION	TIME	CORRECTED ANGLE OF ROTATION
<i>Minutes</i>	<i>Degrees</i>	<i>Minutes</i>	<i>Degrees</i>
0.....	0.00	0.....	0.00
35.....	0.12	35.....	-0.12
90.....	0.25	90.....	-0.25
182.....	0.47	182.....	-0.47
300.....	0.72	300.....	-0.72
428.....	0.93	428.....	-0.94
570.....	-1.12	570.....	-1.11
690.....	-1.22	690.....	-1.24
<i>Hours</i>		<i>Hours</i>	
22.....	-1.48	22.....	-1.48

By comparing the rotations after equal time periods it can easily be seen that the material dried *in vacuo* and redissolved has not lost in any degree whatever in its power to hydrolyze the dipeptid. It seems probable, therefore, that evaporation to dryness *in vacuo* can be applied to preserve this enzyme for an indefinite time, and experiments have been started to see how long the dried material may be kept without losing in activity.

#### PRESENCE OF THE FERMENT AFTER DIGESTION AT 37°.

Knowing that the ferment in solution is gradually destroyed upon standing at 15°, we can assume that the rapidity of this destruction is greater at 37°. Under the influence of sodium chloride and calcium chloride (see below), this has been shown by experiment to be the case. But it is also important to know if the ferment is still present after standing for a number of days in the thermostat at 37°. Five experiments were made with the undialyzed solution, in three of which the ferment was still present after 13 days digestion; in one the test was made after 12 days and in one other after 10 days; in the five cases the presence of the ferment could be demonstrated, for upon the addition of a new portion of the alanyl-glycin the hydrolysis started anew. The optical rotation increased to a certain maximum and remained constant.

The five experiments were all performed in the same way, but only two are given below:

2.50 cc. of the regular solution of racemic alanyl-glycin was mixed with 1.50 cc. of physiological saline solution and 1.00 cc. of the ferment solution and the rate of hydrolysis is given in A. At the end of 13 days the solution was removed from the thermostat. To 2.50 cc. of this material were added 2.50 cc. of a one-half normal solution of racemic alanyl-glycin and the change in the optical rotation is given in B. The concentration of the solution in A was so chosen that at complete hydrolysis the optical rotation of the solution in a 1 dm. tube would be  $-1.51^{\circ}$ . That is, the 2.50 cc. of the hydrolyzed solution A, which had contained before hydrolysis  $1/800$  mol. of racemic alanyl-glycin, were mixed with 2.50 cc. of a one-half normal solution of alanyl-glycin, which also contained  $1/800$  mol. of the racemic dipeptid. The concentration of the material, therefore, after complete hydrolysis was exactly the same as in all the experiments, and we can expect, therefore, a rotation  $-1.51^{\circ}$  to represent the complete hydrolysis of the dipeptid added after 13 days.

SERIES I.

A		B	
2.50 cc. of the normal alanyl-glycin. 1.50 cc. of physiol. saline. 1.00 cc. of pressed yeast juice.		2.50 cc. of the solution in A after 13 days digestion at $37^{\circ}$ . 2.50 cc. of a one-half normal solution of racemic alanyl-glycin.	
TIME	CORRECTED ANGLE OF ROTATION	TIME	CORRECTED ANGLE OF ROTATION
<i>Minutes</i>	<i>Degrees</i>	<i>Minutes</i>	<i>Degrees</i>
0.....	-0.00	0.....	-0.75
33.....	-0.26	40.....	-0.77
47.....	-0.36	<i>Hours</i>	
65.....	-0.50	19½.....	-1.18
93.....	-0.66	25½.....	-1.30
165.....	-1.00	43½.....	-1.47
218.....	-1.17	67½.....	-1.51
285.....	-1.29	<i>Days</i>	
445.....	-1.49	10.....	-1.53
<i>Days</i>			
13.....	-1.51		

SERIES II.

A	B
2.50 cc. of normal racemic alanyl-glycin solution. 1.50 cc. of physiol. NaCl. 1.00 cc. of pressed yeast juice.	2.50 cc. of the solution in A after digesting at $37^{\circ}$ for 12 days. 2.50 cc. of a one-half normal solution of rac. alanyl-glycin.

TIME	CORRECTED ANGLE OF ROTATION	TIME	CORRECTED ANGLE OF ROTATION
<i>Minutes</i>	<i>Degrees</i>	<i>Minutes</i>	<i>Degrees</i>
0.....	-0.00	0.....	-0.76
38.....	-0.82	40.....	-0.82
63.....	-1.25	<i>Hours</i>	
78.....	-1.39	20.....	-1.44
85.....	-1.47	26.....	-1.51
128.....	-1.49	44.....	-1.54
<i>Days</i>		68.....	-1.53
12.....	-1.53	<i>Days</i>	
		10. ....	-1.54

The experiment was repeated with two other fresh enzyme solutions, which were allowed to digest at 37° for 13 days, and with one other fresh enzyme solution from yeast, which was allowed to digest at 37° for 10 days. In these three experiments, which were performed exactly as series I and II the ferment was still present.

The two series given above bring out another fact—they show conclusively that all the dipeptid present in A which the enzyme had the power to hydrolyze had been decomposed after 13 days (respectively 12 days in series II) digestion in the thermostat at 37°.

#### INFLUENCE OF HEAT UPON THE ENZYME SOLUTION.

Although we are justified in assuming that the active principle in pressed yeast juice is thermolabile, still it was considered necessary to decide this point by special experiment. Several experiments were made using both the dialyzed and undialyzed juices.

A. Juice heated alone.—2 cc. of the undialyzed pressed yeast juice were heated in a water bath at a temperature of 75° for a period of 15 minutes. The solution was absolutely clear before being heated, but during the heating became turbid. The test for the presence of the ferment is given below.

B. Juice heated with the alanyl-glycin.—1.00 of the same juice as in A was mixed with 2.50 cc. of a solution of normal racemic alanyl-glycin and 1.50 cc. of physiological saline solution. The mixture was poured into a polariscope tube immediately and the tube with contents heated in a water bath of 76°. A few minutes were required before the contents of the tube had reached a temperature of 75°. The material was heated at this temperature for 15 minutes longer, then cooled to 37° and the optical rotation

determined as shown below. The solution was perfectly clear before heating and but slightly turbid afterwards.

A		B		C	
1.00 cc. of the solution prepared in A. 1.50 cc. of physiol. saline. 2.50 cc. of nor. alanyl-glycin solution.		Solution prepared in B		Control material was not heated.  1.00 cc. of the same enzyme solution used in A and B. 1.50 cc. of physiol. NaCl. 2.50 cc. of the dipeptid solution (alanyl-glycin).	
TIME	CORRECTED ANGLE	TIME	CORRECTED ANGLE	TIME	CORRECTED ANGLE
<i>Minutes</i>	<i>Degrees</i>	immediately after heating	<i>Degrees</i>	<i>Minutes</i>	<i>Degrees</i>
5	0.00			0	0.00
<i>Days</i>				30	-0.10
12	0.00		-0.04	200	-0.70
		<i>Days</i>	-0.07	335	-1.03
		6	-0.06	<i>Hours</i>	
		12		29	-1.47
				45	-1.46

This series proves conclusively that heating the enzyme solution either with the dipeptid or alone for 15 minutes at a temperature of 75° destroys the active principle entirely.

Series II. The enzyme solution subjected to dialysis (6 days

A		B	
TIME	CORRECTED ANGLE OF ROTATION	CONTROL-UNHEATED, SAME MIXTURE AS IN A MT. NOT HEATED.	
		TIME	CORRECTED ANGLE OF ROTATION
immediately after heating	<i>Degrees</i>	<i>Minutes</i>	<i>Degrees</i>
	-0.00	0	0.00
		120	-0.25
<i>Days</i> 9		245	-0.49
	-0.04	370	-0.67
		525	-0.86
		640	-0.99
		<i>Hours</i>	
		21½	-1.37
		<i>Days</i> 6	-1.49



dialysis). A. 2.50 cc. of normal alanyl-glycin solution were mixed with 1.00 cc. of the ferment solution and 1.50 cc. of water and heated in a closed Erlenmeyer flask in a water bath of 75° temperature for six minutes.

The experiment proves that by heating the dialyzed enzyme solution at 75° for six minutes, it has lost its ability to hydrolyze the alanyl-glycin.

#### INFLUENCE OF SODIUM CHLORIDE AND CALCIUM CHLORIDE UPON THE DIALYSED ENZYME SOLUTION.

One of the most important factors in the application of the optical method to the study of enzymes is the one of obtaining an absolutely clear fluid and of avoiding the formation of a precipitate during the time of observation. The early experiments showed that the addition of distilled water in very many cases caused a precipitation probably of some proteids present, and that this precipitation could, in most cases, be avoided by using physiological saline solution instead of water. But whether or not the addition of this salt influenced in any way the rate of hydrolysis was not definitely known. Consequently this has been tested by experiment, using sodium chloride in three different concentrations. The amount of dipeptid used in the four experiments is the same, namely, 2.50 cc. of a normal racemic alanyl-glycin solution, the amount of enzyme was also the same 1.66 cc. of a solution of pressed

	A	B	C	D
TIME	1 PER CENT NaCl	0.1 PER CENT NaCl	0.01 PER CENT NaCl	CONTROL WITHOUT NaCl
	CORRECTED ANGLE	CORRECTED ANGLE	CORRECTED ANGLE	CORRECTED ANGLE
<i>Minutes</i>	<i>Degrees</i>	<i>Degrees</i>	<i>Degrees</i>	<i>Degrees</i>
0	0.00	0.00	0.00	0.00
95	-0.18	-0.20	-0.16	-0.17
228	-0.41	-0.40	-0.39	-0.40
340	-0.59	-0.58	-0.54	-0.54
460	-0.76	-0.75	-0.71	-0.70
615	-0.94	-0.95	-0.90	-0.91
790	-1.11	-1.13	-1.09	-1.08
<i>Hours</i>				
26	-1.42	-1.45	-1.37	-1.39
47	-1.49	-1.51	-1.50	-1.51

yeast juice which had been subjected to dialysis in parchment for six days (designated as "P. Y. J. III<sup>d</sup>" dialyzed). But to mixture of these two was added, in A, 0.84 cc. of a 6 per cent NaCl solution; in B, 0.84 cc. of a 0.6 per cent NaCl solution; in C, 0.84 cc. of a 0.06 per cent NaCl solution; while in D the control, 0.84 cc. of distilled water were added.

By comparing the change in rotation in equal time periods it can be seen that there is but a slight difference, which is, however, almost within the error of the method ( $\pm 0.02^\circ$ ). Since the method itself is not accurate enough to determine the influence of NaCl, another mode of procedure was taken. From various experiments I have been convinced that the action of water alone has the effect of destroying the active principle; even after standing at 15° for ten days the activity of this solution has depreciated noticeably. Consequently the assumption was made that subjecting the enzyme solution to a temperature of 37° for 21 hours would also cause a destruction of the active principle.

Two experiments were made side by side in which the solution of the enzyme was allowed to stand at a temperature of 37° for 21 hours; but to one of these solutions 0.0168 gram of sodium chloride was added before it was placed in the thermostat. The experiment was carried out as follows:

A. 1.66 cc. of the same enzyme solution (P. Y. J. III<sup>d</sup> dialyzed) which was used in the previous experiment, were allowed to stand in the thermostat at a temperature of 37° for 21 hours.

TIME	A	B	C
	ENZYME SOLUTION HEATED TO 37° WITHOUT NaCl FOR 21 HOURS	ENZYME SOLUTION HEATED TO 37° WITH NaCl FOR 21 HOURS	CONTROL-ENZYME SOLUTION ALLOWED TO STAND AT 15° FOR 21 HOURS
	CORRECTED ANGLE OF ROTATION	CORRECTED ANGLE OF ROTATION	CORRECTED ANGLE OF ROTATION
<i>Minutes</i>	<i>Degrees</i>	<i>Degrees</i>	<i>Degrees</i>
0	0.00	0.00	0.00
225	0.17	-0.24	-0.48
400	0.29	-0.43	-0.75
750	0.52	-0.70	-1.12
<i>Hours</i>			
25	0.89	-1.12	-1.40
48	1.25	-1.35	-1.49
<i>Days</i>			
13	1.53	-1.53	-1.50

B. 1.66 cc. of P. Y. J. III<sup>d</sup> dialyzed, were mixed with 0.0168 gram of sodium chloride; after the NaCl had gone into solution the material was allowed to digest at a temperature of 37° for the same time as in A.

C. 1.66 cc. of the same enzyme solution were allowed to stand at a temperature of 15° for 21 hours as a control.

Each of these solutions was then mixed with 0.84 cc. of distilled water and 2.50 cc. of the regular dipeptid solution.

By comparing A and C it can easily be seen that the hydrolytic power of the enzyme has lost considerably after standing at 37° for 21 hours. By comparing A and B, it is also apparent that the sodium chloride has a marked influence in preserving the enzyme. The destruction influence of water alone is, therefore, prevented to a marked degree by the presence of sodium chloride. It seems, however, too soon to make any speculation as to how this is brought about.

#### INFLUENCE OF CALCIUM CHLORIDE.

Delezenne<sup>1</sup> has shown that calcium salts have the property of activating pancreatic juice. It was consequently of importance to study the influence of a calcium salt upon the action of pressed yeast juice. Two series of experiments were made with the same P. Y. J. III<sup>d</sup>, which had been subjected to dialysis for six days (it contained 0.45 per cent solids), as was used in the two previous experiments. The influence of calcium chloride was studied in a 1.0 per cent, 0.1 per cent and 0.01 per cent solution.

To 2.50 cc. of the regular solution of alanyl-glycin and 1.66 cc. of the enzyme solution were added, in A. 0.84 cc. of a 6 per cent CaCl<sub>2</sub> solution, in B. 0.84 cc. of a 0.6 per cent Ca Cl<sub>2</sub> solution, in C. 0.84 of a 0.06 per cent CaCl<sub>2</sub> solution, and in D. the control, 0.84 cc. of water. All four mixtures were digested as usual at a temperature of 38° during the time of observation.

It can be seen plainly that the hydrolysis of the dipeptid is more rapid in a 0.1 per cent solution of calcium chloride. Both series show this definitely. While in a 1 per cent solution of calcium chloride the hydrolysis is less rapid than in the control D, in which no calcium chloride has been added. This is indeed

<sup>1</sup> Delezenne: *Compt. rend. soc. biol.*, 1907, ii, p. 274.

SERIES I

TIME	A	B	C	D
	1 PER CENT CaCl <sub>2</sub>	0.1 PER CENT CaCl <sub>2</sub>	0.01 PER CENT CaCl <sub>2</sub>	No CaCl <sub>2</sub> AS CONTROL
	CORRECTED ANGLE OF ROTATION	CORRECTED ANGLE	CORRECTED ANGLE	CORRECTED ANGLE
Minutes	Degrees	Degrees	Degrees	Degrees
0	0.00	0.00	0.00	0.00
120	-0.25	-0.25	-0.24	-0.25
245	-0.46	-0.51	-0.50	-0.49
370	-0.64	-0.71	-0.67	-0.67
525	-0.81	-0.92	-0.88	-0.86
640	-0.92	-1.05	-1.02	-0.99
Hours				
21½	-1.24	-1.42	-1.44	-1.37
Days				
6	-1.48	-1.49	-1.50	-1.49

SERIES II

Exactly the Same as Series I

TIME	A	B	C	D
	1 PER CENT CaCl <sub>2</sub>	0.1 PER CENT CaCl <sub>2</sub>	0.01 PER CENT CaCl <sub>2</sub>	No CaCl <sub>2</sub>
	CORRECTED ANGLE	CORRECTED ANGLE	CORRECTED ANGLE	CORRECTED ANGLE
Minutes	Degrees	Degrees	Degrees	Degrees
0	0.00	0.00	0.00	0.00
145	-0.33	-0.34	-0.33	-0.32
270	-0.52	-0.58	-0.57	-0.53
390	-0.70	-0.78	-0.75	-0.71
510	-0.83	-0.95	-0.91	-0.87
645	-0.94	-1.10	-1.05	-1.02
775	-1.03	-1.22	-1.17	-1.13
Hours				
25	-1.33	-1.48	-1.47	-1.43
32	-1.38	-1.49	-1.50	-1.46
48	-1.43	-1.49	-1.51	-1.50
Days				
16	-1.48			-1.50

remarkable. In the series with sodium chloride it can easily seen that there is no inhibition of the hydrolysis in a 1 per cent lution—where the ion concentration is about the same. It see

therefore, that the calcium chloride itself has some specific action in inhibiting in a 1 per cent solution.

#### SUMMARY.

For the study of the proteolytic enzymes, racemic alanyl-glycin can be applied with great accuracy, using the optical method.

Solutions of d-alanyl-d-alanin and of racemic alanyl-glycin remain unchanged when they are allowed to stand at 15°–20° for a period of 13 months, if toluol has been used as preservative. This proves that within the time mentioned neither water nor bacteria have any influence in hydrolyzing the dipeptids.

Buchner's grinding and pressing method yields the most active enzyme.

The precipitation with alcohol can not be used to advantage in the purification of the active principle.

The active principle which hydrolyzes alanyl-glycin has the property of dialyzing through parchment. The solution of the enzyme which has been freed from most of the solids by dialysis can be evaporated to dryness and redissolved without being impaired in its activity.

The ferment is still present after 13 days' digestion at 37°.

Heating the solution of the enzyme to a temperature of 75° for six minutes destroys the active principle completely.

Sodium chloride has no influence upon the rate of hydrolysis. It has, however, the property of preserving the enzyme if digested at 37° for 21 hours. Calcium chloride has the property of increasing slightly the rate of hydrolysis in a 0.1 per cent solution, while it inhibits markedly in a 1 per cent solution.



# SOME PECULIARITIES OF THE PROTEOLYTIC ACTIVITY OF PAPAÏN.

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## THE ACCELERATION OF PROTEOLYSIS BY HYDROCYANIC ACID.

The enzyme contained in the latex of *Carica papaya*, and generally known as papain, has been studied far more than any other vegetable proteolytic enzyme. Nevertheless, the fundamental question whether or not papain carries digestion of protein to the amino-acid stage has received antagonistic answers even by recent investigators. Methods of separating digestion products, while they lack the precision of inorganic procedures, are yet reasonably

satisfactory; and it would seem that it ought to be possible to answer this question definitely. Vines has suggested that the puzzling discrepancies on record are due to the use of different antiseptics by different investigators, and that the common preservatives have more specific action than is ordinarily supposed. Since Vines's criticism can be understood only in its historical connection, a brief review of the literature is necessary.

Wurtz, who was the first to subject papain to well controlled scientific study, reported so-called leucine<sup>1</sup> among the products of digestion and classed this enzyme with trypsin. Subsequently, Martin<sup>2</sup> obtained "leucine" in the alcoholic extract from the precipitation of albumoses and peptones, both from fibrin digestions and from the hydrolysis of the proteins obtained from the dried pawpaw milk itself. He did not obtain any crystalline tyrosine, but concluded that it was formed since the residues from the evaporation of the alcoholic extract, which gave no biuret reaction, did give the Millon's test. Chittenden<sup>3</sup> reported the formation of large proportions of peptone together with small amounts of leucine and tyrosine from the digestion of coagulated egg-white, fibrin, and raw and cooked meat. The following year Sharp<sup>4</sup> and Dott<sup>5</sup> denied the formation of peptone in digestions of coagulated egg-white and serum albumin. Chittenden, Mendel and McDermott<sup>6</sup> substantiated Chittenden's results showing that the chief products of papain digestion were deuto-albumose and peptone. "Even under the most favorable conditions the formation of amino-acids or other crystalline decomposition products is extremely slight." Harlay,<sup>7</sup> who studied the milk from *Carica hastifolia*, a species closely related to *Carica papaya*, found that it resembled pepsin rather than trypsin in the behavior of its digestion products

<sup>1</sup> Wurtz: *Compt. rend. de l'acad. des sci.*, xci, p. 788, 1880.

<sup>2</sup> Martin: *Journ. of Physiol.*, v, p. 213, 1884; vi, p. 336, 1885.

<sup>3</sup> Chittenden: *Trans. Conn. Acad. of Arts and Sci.*, ix, p. 298, 1892.

<sup>4</sup> Sharp: *Pharm. Journ. Trans.*, liii, p. 637, 757; See *Maly's Jahresb.*, xxiv, p. 318, 1894.

<sup>5</sup> Dott: *Pharm. Journ. Trans.*, liii, 758; See *Chem. Centralbl.*, i, p. 831 1894.

<sup>6</sup> Chittenden, Mendel, and McDermott: *Amer. Journ. of Physiol.*, i, p. 255, 1898.

<sup>7</sup> Harlay: *Compt. rend. soc. de biol.*, lii, p. 112, 1900; *Journ. de pharm. et chim.*, 6<sup>e</sup> Ser., xi, p. 172, 1900.



with tyrosinase (from *Russula delica*). In 1901 Vines<sup>1</sup> incidentally reported positive tryptophane tests with chlorine water in papain digestions of fibrin. The same year Mendel and Underhill<sup>2</sup> in over sixty digestion experiments, using four different samples of papain, and casein, fibrin, coagulated egg-white and boiled and raw muscle as substrata, obtained evidence of leucine, tyrosine and tryptophane with uncoagulated muscle only. This exception they attributed to autolytic action. As Mendel<sup>3</sup> pointed out, the results of Vines cited above are open to the objection that no antiseptic was used, and the possibility of the bacterial origin of the tryptophane was not excluded. Vines,<sup>4</sup> in subsequent experiments, corrected this fault by the use of HCN. In replying to Mendel and Underhill, he<sup>5</sup> attributed the failure of these authors to obtain amino-acids to their use of NaF—an antiseptic which he regards as peculiarly unfavorable to papain proteolysis. In 1902, Emmerling<sup>6</sup> obtained small amounts of arginine, tyrosine, leucine, aspartic acid, glycocoll, alanine and phenylalanine from a six-weeks' digestion of fibrin in which the papain was added in fractions during the course of the digestion. In 1905, Vines<sup>7</sup> came to the conclusion that various commercial papains differ from each other considerably in their behavior toward acidity and alkalinity and antiseptics, but he invariably obtained tryptophane tests from peptone digestions made under suitable conditions. That the amount of amino-acids obtained by Emmerling was not larger, Vines attributed to a small concentration of papain and to a peculiarly unfavorable combination of conditions, chief among which was the use of toluene as an antiseptic. Kutscher and Lohmann<sup>8</sup> in 1905, obtained arginine, lysine, tyrosine, leucine and valine from a ten months' digestion of fibrin with papain, using chloroform as antiseptic. A year later Abderhalden and Teruuchi<sup>9</sup>

<sup>1</sup> Vines: *Annals of Botany*, xv, p. 570, 1901.

<sup>2</sup> Mendel and Underhill: *Trans. Conn. Acad. of Arts and Sci.*, xi, p. 1, 1901.

<sup>3</sup> Mendel: *Amer. Journ. of Med. Sci.*, cxxiv, p. 310, 1902.

<sup>4</sup> Vines: *Annals of Botany*, xvi, p. 5, 1902.

<sup>5</sup> Vines: *Annals of Botany*, xvii, p. 602, 1903.

<sup>6</sup> Emmerling: *Ber. d. deutsch. chem. Ges.*, xxxv, p. 695, 1012, 1902.

<sup>7</sup> Vines: *Annals of Botany*, xix, p. 149, 1905.

<sup>8</sup> Kutscher and Lohmann: *Zeitsch. f. physiol. Chem.*, xlvi, p. 383, 1905.

<sup>9</sup> Abderhalden and Teruuchi: *Zeitsch. f. physiol. Chem.*, xlix, p. 21, 1906.

reported that papain hydrolyzed glycyl-l-tyrosine; but the amounts of amino-acids which they recorded were rather small.<sup>1</sup> From a study of the data, it appears, leaving Vines's papers out of consideration for the moment, that the formation of amino-acids was as a rule small, even when a large proportion of papain was used and digestion was prolonged. This is true when the papain formed albumoses and peptones rapidly,<sup>2</sup> and the results cannot therefore be attributed to a low concentration of enzyme in the papain preparations.

In the light of these conflicting results, Vines's contention that proteolysis by this enzyme is more complete in the presence of HCN than of the other common antiseptics deserves consideration. His conclusion was based on the depth of the tryptophane test obtained when Witte's peptone or fibrin was digested with papain in the presence of HCN, sodium fluoride and chloroform respectively. In the case of fibrin the rate of solution was also observed. "It appears that neither chloroform nor sodium fluoride inhibits the peptonizing action of papain, but that they both impede further proteolysis with the formation of tryptophane." The experiments "strikingly demonstrate the remarkably favorable effect of the presence of HCN upon the proteolytic activity, as also the inhibitory effect of sodium fluoride."<sup>3</sup> In a more extended series of experiments Vines found that salicylic acid, thymol, toluene and formalin fall in the same class with chloroform and sodium fluoride.

In discussing the observations and deductions of this author three points must be kept in mind. 1. Hydrocyanic acid was the only antiseptic in the presence of which papain split off any considerable amounts of tryptophane from fibrin or from Witte's peptone. 2. The difference between HCN and other anti-

<sup>1</sup> These different investigators have used various commercial preparations of papain. Emmerling, Kutscher and Lohmann, and Abderhalden and Teruuchi, worked with Merck's "papayotin." Vines studied the products put out by Merck, Finkler, and Christy respectively. Chittenden used papain sold by the American firm of Johnson and Johnson under the name "papoid." Mendel and Underhill worked with "caroid," "papoid" and two samples of "papain" from the firms of Lehn and Fink, and Merck respectively.

<sup>2</sup> Cf. Chittenden, Mendel and McDermott: *loc. cit.*

<sup>3</sup> Vines: *Annals of Botany*, xvii, p. 606, 1903.

septics was assigned to two causes: acceleration by HCN; and inhibition by chloroform, toluene, sodium fluoride, etc. 3. These antiseptics are said to exert their specific influence only on the later phases of digestion. In a subsequent paper Vines adduced this difference in the action of antiseptics in the early and late steps in the hydrolysis of protein in support of the view that papain is not a single enzyme but a mixture of a peptonizing enzyme and an erepsin.

"The behaviour of papain in the presence of sodium fluoride may be explained, on the assumption of a single tryptic enzyme, by supposing that this protease is so acted upon by sodium fluoride that its peptolyzing activity is inhibited; its peptonizing, fibrin-digesting activity remaining unimpaired ... This supposition is less reasonable than the assumption that two distinct proteases are present and that the arrest of the peptolytic action of papain by sodium fluoride is due to the inhibition of the peptolytic enzyme (erepsin)."<sup>1</sup>

If Vines's observations are valid, the major discrepancies concerning the products of papain proteolysis noted in the literature are explicable. The early workers, Wurtz<sup>2</sup> and Martin<sup>3</sup> who reported leucine and leucine and tyrosine respectively, used small amounts of HCN as an antiseptic. Chittenden and all subsequent investigators who have used thymol, sodium fluoride, toluene or chloroform have either failed to obtain amino-acids or obtained them in fair quantity only after long digestion.

Turning to a consideration of the explanation of the action of different antiseptics given by Vines, it is a little surprising to have marked inhibitory powers attributed to toluene, which is usually considered the least harmful of antiseptics.<sup>4</sup> Although in his earlier papers Vines has called attention to the possible favorable influence of HCN in distinction from the other substances used, this feature seems to have been lost sight of in the later discussion. Here, it will be noted, the inhibitory factor alone receives emphasis (cf. p. 181 above).

There is little in the literature to lead one to expect a pronounced acceleration of proteolysis by HCN. It was a common antiseptic

<sup>1</sup> Vines: *Annals of Botany*, xix, p. 174, 1905.

<sup>2</sup> Wurtz: *loc. cit.*

<sup>3</sup> Martin: *loc. cit.*

<sup>4</sup> Cf. Oppenheimer's *Die Fermente und ihre Wirkungen*, p. 41, 1900.

in the early days, but was entirely abandoned for sodium fluoride, thymol, chloroform and toluene. Wurtz and Martin both used it in their studies on papain; and Green employed KCN in his work on the Kachree gourd (*Cucumis utilisissimus*, Roxb.).<sup>1</sup> Fiechter<sup>2</sup> recorded that HCN began to affect pepsin and trypsin only at such high concentrations that the acidity might be held responsible. Chittenden and his pupils found that potassium cyanide retarded peptic digestion<sup>3</sup> but accelerated tryptic digestion. Butkewitsch quotes Loew<sup>4</sup> to the same effect. Hahn and Geret<sup>5</sup> reported that 1 per cent HCN only slightly inhibited the digestion of egg-white by yeast endotrypsin. Quite recently, Abderhalden, Cammerer and Pincussohn<sup>7</sup> have found that low concentrations of potassium cyanide distinctly favor, and high concentrations distinctly inhibit, the hydrolysis of certain dipeptides by yeast endotrypsin. Far the most interesting observation is that of Butkewitsch<sup>8</sup> on the effect of chloroform, toluene and HCN respectively on the autolysis of seedlings of the lupine (*Lupinus luteus*). He says that "HCN, especially in concentrations of 0.2 per cent, caused a conspicuous increase in the amount of product precipitable by phosphotungstic acid." Since 0.2 per cent HCN had a similar effect (though not so marked) he thought that the acceleration might be attributed to acidity. According to Vines, HCN gives distinctly better results than other antiseptics with many proteolytic enzymes of plant origin.

Our own preliminary experiments demonstrated that the contrast between the amount of tryptophane which appears in papain-peptone digestions with HCN and with other antiseptics respectively is as striking as Vines stated. They indicated, however, that the difference is due to a marked acceleration by HCN.

<sup>1</sup> Green: *Annals of Botany*, vi, p. 195, 1892.

<sup>2</sup> Fiechter: *Ueber den Einfluss der Blausäure auf Fermentvorgänge*, dissertation, Basel, 1875; see *Maly's Jahresb.*, v, p. 269, 1875.

<sup>3</sup> Chittenden and Allen: *Studies from the Laboratory of Physiological Chemistry, Sheffield Scientific School*, p. 87, 1884-1885.

<sup>4</sup> Chittenden and Cummins: *Ibid.*, p. 107.

<sup>5</sup> Loew: *Die chemische Energie der lebenden Zellen*, München, p. 149, 1898.

<sup>6</sup> Hahn and Geret: *Zeitschr. f. Biol.*, xl, p. 144, 1900.

<sup>7</sup> Abderhalden, Cammerer and Pincussohn: *Zeitschr. f. physiol. Chem.*, lix, p. 293, 1909.

<sup>8</sup> Butkewitsch: *Zeitschr. f. physiol. Chem.*, xxxiii, p. 24, 1901.

an acceleration far more pronounced than anything in the literature would lead one to expect. Experiments were undertaken to discover if the acceleration by HCN is limited to the "ereptic" activity and to throw some light on the mechanism of the action of this antiseptic.<sup>1</sup>

DESCRIPTION OF THE PAPAÏN USED IN THESE EXPERIMENTS. Through the kindness of Mr. William Harris of the Hope Botanical Gardens, Jamaica, we were fortunate enough to obtain from Mr. F. G. Sharp a sample of papain which had an advantage over commercial preparations in that its history was at least partially known. The material consisted of sun-dried latex of the *Carica papaya*, collected in December, when the temperature to which it was exposed did not, according to Mr. Sharp, exceed 40° C. The sample was not over a month old when the following experiments were begun.

This papain powder was not entirely soluble in a hundred times its weight of 1 per cent sodium chloride solution; but that weight of solvent extracted nearly all the enzyme. For experiments a freshly prepared solution of this strength, filtered clear, was used, unless otherwise specified. The saline extract was barely acid to litmus, gave a distinct biuret reaction, but no tryptophane test, and developed no tryptophane when autolyzed with toluene or HCN as antiseptic.

*The "Ereptic" Activity of Papain in the Presence of Various Antiseptics.*

It is obviously impossible to arrive at any conclusion as to whether an antiseptic does or does not inhibit proteolysis by comparing a digest made in its presence with one which contains no antiseptic, unless the antiseptic-free digest is *aseptic*. Since it was not convenient to employ a strictly aseptic technique, a different method was adopted—namely, that of comparing digests containing various antiseptics with a series containing HCN in addition. If toluene inhibits proteolysis, it might be expected to prevent the formation of tryptophane even when HCN is present. If, on the other hand, toluene is inert, but HCN accelerates

<sup>1</sup>The experimental data are taken from the thesis presented by Alice F. Blood for the degree of Doctor of Philosophy, Yale University, 1910.

digestion, proteolysis should be marked when b  
are used. The following experiment was pla  
principle.

THE FORMATION OF TRYPTOPHANE FROM WITTE'S PEI  
5 per cent Witte's peptone in 1 per cent NaCl solution; 3 cc.  
in 1 per cent NaCl solution; antiseptics as indicated in  
volume, 6.6 cc.; digested in glass-stoppered bottles at 4  
The two series were tested for tryptophane by adding sa  
water drop by drop to 2 cc. portions of the digests. A con  
no tryptophane was liberated from Witte's peptone by HCN  
conditions of the experiment.

Digestion of Witte's Peptone in the Presence of Variou

ANTISEPTICS	TRYPTOPHANE	
	Without HCN	With
Toluene.....	None	
Chloroform, 0.5 per cent.....	None	
Thymol, 0.5 per cent.....	None	
Sodium fluoride, 1 per cent.....	None	
Boric acid, 0.3 per cent .....	None	
None, .....	.....	

From an inspection of the data it is clear that in  
HCN conspicuous amounts of tryptophane are l  
Witte's peptone by concentrations of papaïn which g  
of the formation of this amino-acid in the presen  
chloroform, thymol, sodium fluoride or boric aci  
as these antiseptics do not prevent the formation of  
HCN is present simultaneously, it appears that t  
*ference in digestion in the presence of HCN and o*  
*respectively is due to acceleration of proteolysis by l*

Although no sign of “ereptic” activity was obtai  
conditions just cited, unless HCN was present, evi  
tained that tryptophane was liberated slowly in t  
HCN when much larger proportions of enzyme w  
toluene for antiseptic) and digestion was continued  
more.

**THE FORMATION OF LEUCINE AND TYROSINE FROM COAGULATED EGG-WHITE.** The marked accelerating action of HCN on the final stages of papain proteolysis having thus been demonstrated, it became important to search for other amino-acids under the most favorable conditions for proteolysis. Parallel digestions of coagulated egg-white, with and without HCN, indicated that leucine and tyrosine as well as tryptophane are readily formed in the presence of HCN.

The whites of a dozen eggs were coagulated in a flocky condition by the usual procedure of pouring them into a large volume of boiling water faintly acidulated with acetic acid. After washing the coagulum repeatedly by decantation until it was no longer acid to litmus, it was drained and divided into two parts of 135 gms. To each portion suspended in 500 cc. of water were added 200 cc. of a 2 per cent extract of papain in 1 per cent saline, and toluene. To one portion was added also 70 cc. of 1.7 per cent HCN (concentration, approx. 0.15 per cent.) Both mixtures were digested in sealed jars for a week at 40°.

Mer inspection showed that HCN markedly accelerated the rate of solution of the egg white. In the digest containing this antiseptic nearly all of the protein was dissolved within 5 hours; in the other, 50 gms. were still undissolved at the end of the week. 2 cc. portions of solution were removed daily and tested for tryptophane. In the digest containing HCN, a strong reaction was given at the end of 24 hours; in the other, no evidence of tryptophane was obtained within a week.

After a week, both digests were filtered and evaporated to about 75 cc. Large amounts of "leucine" and tyrosine separated from the digest containing HCN; none were thus obtained from the parallel digest which did not contain this antiseptic.

### *Is the Acceleration by Hydrocyanic Acid Limited to the "Ereptic" Activity of Papain?*

The previous experiment indicated that HCN favors the early stages of the digestion of protein as well as the final cleavage into amino-acids. Especial interest therefore attaches to the study of the effect of different antiseptics on the digestion of uncoagulated egg-white, a protein mixture, which according to the work of Delezenne, Mouton and Pozerski<sup>1</sup> is extremely resistant to digestion by papain at 40°.

<sup>1</sup> Delezenne, Mouton and Pozerski: *Compt. rend. soc. de biol.*, lx, p. 309, 1906.



**THE DIGESTION OF UNCOAGULATED EGG-WHITE.** A solution of egg-white was prepared by diluting slightly beaten white of egg with three volumes of 1 per cent sodium chloride solution and filtering the mixture. The amount of protein undigested at the end of the experiment was determined by the trichloracetic acid method, applied by Martin<sup>1</sup> and recently used by Delezenne, Mouton and Pozerski.<sup>2</sup>

Fifteen cc. egg-white solution; 1 cc. 1 per cent papaïn solution; antiseptics as indicated in the table; total volume 30 cc.; digested at 40° for 17 hours. The undigested protein was precipitated with 30 cc. of a 10 per cent solution of trichloracetic acid; the mixture was heated for 5 minutes in a 100° water bath to completely coagulate the protein and dissolve any albumoses, and filtered while hot through a dried tared filter paper. The coagulum was washed free from acid with water and dried to constant weight. The amount of protein in the original mixture of egg-white and papaïn was determined in a blank by the trichloracetic acid method.

*Digestion of Uncoagulated Egg-white in the Presence of Various Antiseptics.*  
(15 cc. egg + 1 cc. papaïn = 0.399 gram protein.)

ANTISEPTIC	PROTEIN DIGESTED	
	Without HCN	With HCN (.15 per cent)*
	gram	gram
Toluene.....	0.036	0.291
Sodium fluoride, 1 per cent.....	0.013	0.220
Chloroform, 0.5 per cent.....	0.016†	0.227
Boric acid, 0.3 per cent.....	0.061	0.299
None.....	.....	0.290

\* A control showed that HCN does not itself hydrolyze the proteins of egg-white under the conditions of the experiment.  
† In other experiments the digestion was distinctly greater in the presence of chloroform than of toluene, sodium fluoride or boric acid.

Although there are minor differences in the amount of digestion in the presence of toluene, chloroform, sodium fluoride and boric acid<sup>3</sup> respectively, these are insignificant in comparison with the relative rapidity with which egg-white is digested by papaïn in

<sup>1</sup> Martin: *Journ. of Physiol.*, xv, p. 375, 1894.  
<sup>2</sup> Delezenne, Mouton and Pozerski: *loc. cit.*  
<sup>3</sup> The experiments on the rate of digestion of egg-white at 100° in the presence of various antiseptics and without any antiseptics (cf. p. 203) furnish additional evidence that none of the substances in question exert any marked inhibition over papaïn proteolysis.



the presence of HCN, even when these other antiseptics are also present. The data entirely confirm the conclusion reached in the experiment on Witte's peptone that *the difference in the amount of digestion in the presence of HCN and other antiseptics is due primarily to a very pronounced acceleration of proteolysis by HCN.*

Observations on the effect of HCN on the rate of fibrin solution, of gelatin liquefaction, and of milk clotting indicate that *HCN accelerates even those early stages of hydrolysis which papain is able to accomplish rapidly without it.*<sup>1</sup>

**THE SOLUTION OF FIBRIN.** A small flock of fibrin; 5 cc. 1 per cent papain in 1 per cent NaCl solution; acid, alkali and HCN as indicated in the table; toluene; examined after 5 hours digestion at 40°.

*The Solution of Fibrin by Papain With and Without HCN.*

ACID OR ALKALI	CONDITION OF FIBRIN	
	Without HCN*	With HCN (0.15 per cent)
0.5 cc. $\frac{N}{10}$ Na <sub>2</sub> CO <sub>3</sub> .....	No apparent disintegration	Almost completely dissolved
0.5 cc. $\frac{N}{10}$ HCl .....	No apparent disintegration	Almost completely dissolved
None.....	No apparent disintegration	Almost completely dissolved

\* After 24 hours digestion the fibrin was well broken up but not completely dissolved in the three digests which contained no HCN.

**THE LIQUEFACTION OF GELATIN.** The influence of HCN on the liquefaction of gelatin by papain was determined by finding the amount of enzyme, with and without this antiseptic, necessary to hydrolyze the same amount of gelatin under definite conditions to a point at which it no longer solidified on cooling.

Two cc. 5 per cent "melted" gelatin (0.1 per cent thymol); one series with, one without, HCN (0.15 per cent); water to make a final volume of 4 cc.; 1 per cent papain solution in amounts indicated in the table; digested at 40° for 15 minutes; chilled and examined for solidification.

<sup>1</sup> The marked acceleration of the rate of solution of fibrin by papain produced by HCN, although perfectly clear in Vines's data, seems to have been overlooked by him. Cf. Vines: *Annals of Botany*, xvii, p. 605, 1903.

*Liquefaction of Gelatin by Papaïn With and Without HCN.*

1 PER CENT PAPAÏN SOLUTION	CONDITION OF GELATIN	
	Without HCN	With HCN (.15 per cent)
cc.		
0.05.....	Solid	Solid
0.10.....	Solid	Liquid
0.15.....	Solid	Liquid
0.20.....	Solid	Liquid
0.25.....	Liquid	Liquid
0.35.....	Liquid	Liquid
0.50.....	Liquid	Liquid
None.....	Solid	Solid

THE CLOTTING OF MILK. Milk from which most of the fat had been removed by skimming and subsequent extraction with chloroform was used. The method employed was the same in principle as that described in the experiment on gelatin. The amount of enzyme, with and without HCN, necessary to clot a certain amount of milk under definite conditions was determined.

Three cc. milk; one series with, one without, HCN (0.15 per cent); water to make a final volume of 4 cc.; 1 per cent papaïn solution in the amounts indicated in the table. The papaïn was added rapidly, at the last moment before digesting the mixture, to the chilled diluted milk. The table shows the condition of the milk after warming for 10 min. at 40°.

*Clotting of Milk by Papaïn With and Without HCN.*

1 PER CENT PAPAÏN SOLUTION	CONDITION OF MILK	
	Without HCN	With HCN (0.15 per cent)
cc.		
0.05.....	Unclothed	Clotted
0.10.....	Unclothed	Clotted
0.15.....	Unclothed	Clotted
0.20.....	Unclothed	Clotted
0.25.....	Unclothed	Clotted
0.30.....	Unclothed	Clotted
0.35.....	Unclothed	Clotted
0.40.....	Unclothed	Clotted
0.45.....	Clotted	Clotted
None.....	Unclothed	Unclothed

It might be argued that the beneficial effect of HCN on the liquefaction of gelatin and the clotting of milk by papain is due to the hydrogen ions of the antiseptic. The experiment on the acceleration of the solution of fibrin in media which are acid with hydrochloric acid and alkaline with sodium carbonate indicates that the explanation of the action of HCN is not so simple. This point was tested thoroughly on both Witte's peptone and egg-white (cf. p. 192).

**THE DIGESTION OF VEGETABLE PROTEINS.** Experiments on the digestion by papain of excelsin and edestin<sup>1</sup> showed that the hydrolysis of typical plant proteins into products not precipitated by trichloroacetic acid<sup>2</sup> is also greatly favored by HCN.

A solution of *edestin* was prepared by dissolving 2 grams of this protein in 75 cc. of 10 per cent sodium chloride solution. An undissolved residue was filtered off and the amount of proteins was determined by precipitating with hot trichloroacetic acid (cf. p. 186). 10 cc. edestin solution; 10 cc. 1 per cent papain solution; one digest with, one without, HCN; total volume 25 cc. made up with 10 per cent NaCl solution (final concentration of sodium chloride about 5 per cent); toluene; digested at 40° for 17 hours. The undigested edestin was determined by the trichloroacetic acid method. The mixture of edestin and papain contained 0.21 gram of protein precipitable by trichloroacetic acid. In the digest containing toluene 0.07 gram was digested; in that containing HCN in addition 0.20 gram was digested.

A solution of *excelsin* was prepared by titrating a suspension of this protein in water, to the point of neutrality to phenolphthalein. Otherwise the experiment was similar to that on edestin. The mixture of excelsin and papain contained 0.27 gram of protein precipitable by trichloroacetic acid. In the digest containing toluene, 0.06 gram was digested; in that containing HCN in addition, 0.23 gram was digested.

### *Is "Papain" a Mixture of Proteolytic Enzymes?*

Inasmuch as HCN facilitates the early phases of proteolysis as well as the final cleavage into amino-acids, the action of this antiseptic cannot be adduced, as Vines has done, as a proof that papain consists of two enzymes, a pepsin and an erepsin, unless one assume

<sup>1</sup> For the samples of these plant proteins we are greatly indebted to Dr. T. B. Osborne.

<sup>2</sup> These experiments, though cast in a quantitative form, are to be taken only in a qualitative sense. Trichloroacetic acid does not precipitate these plant proteins quantitatively under the conditions recorded.

that the early stages of digestion are benefited only indirectly through the constant reduction in the concentration of the products of pepsin-proteolysis by the activity of the erepsin. Vines has recently reported a partial separation of the proteolytic enzymes of papain, making use of the differential solubility in sodium chloride solution. According to him, if papain is extracted with twenty times its weight of 2 per cent sodium chloride solution, part of the peptonizing enzyme and all of the erepsin are extracted. If the residue is washed with water and again extracted with 2 per cent sodium chloride solution, an extract is obtained which dissolves fibrin but does not liberate tryptophane from Witte's peptone. (HCN was used as antiseptic.)

From a careful repetition of the experiment described by Vines, it appears to us that the results are attributable to dilution rather than to a separation of enzymes. If the first saline extract was diluted with 100 volumes of 2 per cent sodium chloride solution a solution was obtained whose properties were indistinguishable from the second saline extract. Both disintegrated and partially dissolved fibrin in two days, but neither gave any evidence of the formation of tryptophane in that time.

The extreme differences in the rate at which papain accomplishes the earlier and the later steps in digestion (in the absence of HCN) furnish strong presumptive evidence that "papain" is a mixture of at least two enzymes; but conclusive proof that this is the case has not as yet been furnished.

#### THE MECHANISM OF THE ACCELERATION OF PAPAÏN PROTEOLYSIS BY HYDROCYANIC ACID.

Several possible explanations of the mode of action of HCN in accelerating the hydrolysis of protein by papain suggest themselves. Is it not accounted for by the concentration of hydrogen ions from the acid? Is it due rather to a specific action of the cyanide radical? Does HCN destroy inhibiting substances in the papain or in the substrata? Does it activate a papain zymogen as lime salts activate trypsinogen? The experiments which follow attempt to answer these and related questions.

<sup>1</sup> Vines: *Annals of Botany*, xxiii, p. 1, 1909.

**METHODS.** The two methods chosen for studying the mechanism of the action of HCN were the digestion of Witte's peptone as indicated by the liberation of tryptophane and the digestion of white of egg as judged by the conversion into products which are not precipitable by trichloroacetic acid. The details of the procedure have already been given (p. 184 and p. 186). These two methods differ from each other in several important respects. 1. One is a test of the early, the other of the later phases of digestion. 2. In Witte's peptone, the protein has been subjected to a fairly drastic treatment and may be supposed to have lost any "native resistance" to digestion such as raw egg-white in particular is supposed to show. 3. The amount of digestion of egg-white admits of a better quantitative expression than the depth of the tryptophane test. The terms in which one can describe the latter—faint, distinct, marked, strong, deep—have a purely relative meaning, determined by the range of colors with which one happens to be dealing. Both methods have the advantage that in the absence of HCN digestion is practically lacking.

*Is the Acceleration by Hydrocyanic Acid due to the Concentration of Hydrogen Ions?*

The most obvious explanation of the action of HCN is that it furnishes a concentration of hydrogen ions peculiarly favorable to the action of papain. If this is the explanation, equivalent concentrations of acids of similar dissociation should give like results. It must be remembered in this connection that HCN is one of the weakest acids whose dissociation has been measured. According to Walker<sup>1</sup>, boric acid, if considered as a monobasic acid, has almost exactly the same dissociation as HCN, and hydrogen sulphide is only slightly stronger. The following experiments show the comparative effect of these acids.

Three cc. 5 per cent Witte's peptone solution; 3 cc. 1 per cent papain solution; equivalent amounts of the acids indicated in the table; total volume 6.6 cc.; toluene; digested at 40° for 17 hours. Before testing for tryptophane, the hydrogen sulphide was removed by boiling.

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<sup>1</sup> Walker: *Zeitschr. f. physikal. Chem.*, xxxii, 137-141, 1900.

*The Digestion of Witte's Peptone in the Presence of Very Weak Acids.*

ACID	TRYPTOPHANE TEST
Hydrocyanic, 0.15 per cent .....	Strong
Boric, 0.32 per cent .....	None
Hydrogen sulphide (Peptone saturated)*...	Marked

\* The concentration of hydrogen sulphide, although not strictly equivalent, is of the same order of magnitude.

Fifteen cc. egg white solution; 10 cc. 1 per cent papain solution; equivalent amounts of the acids indicated in the table; total volume 30 cc.; toluene; digested at 40° for 17 hours.

*The Digestion of Raw Egg-white in the Presence of Very Weak Acids.*  
(15 cc. egg-white + 10 cc. papain = 0.470 gram protein.)

ACID	PROTEIN DIGESTED
	gram
Hydrocyanic, 0.15 per cent .....	0.415
Boric, 0.32 per cent .....	0.061
Hydrogen sulphide (egg solution saturated)	0.356

Of these two acids which have practically the same dissociation as HCN, one alone resembles it in accelerating proteolysis. The influence of HCN can, therefore, hardly be ascribed to its acidity. It might be argued that in boric acid the acceleration due to the hydrogen ions is overbalanced by a specific inhibition exerted by the boric ions or by undissociated boric acid. Inasmuch as it has already been shown (p. 186) that boric acid does not interfere with the acceleration produced by HCN, this view does not seem tenable. That the phenomenon in question is not due to the concentration of hydrogen ions is indicated, furthermore, by the following experiments in which the effect of HCN was determined over a considerable range of acidity and alkalinity.

Three cc. 5 per cent Witte's peptone solution; 3 cc. 1 per cent papain solution; acid, alkali, and HCN as indicated in the table; total volume 7 cc.; toluene; digested at 40° for 17 hours.

Fifteen cc. egg-white solution; 5 cc. 1 per cent papain solution; acid, alkali and HCN as indicated in the table; total volume 30 cc.; toluene; digested at 35° for 19 hours.

*The Acceleration of the Digestion of Witte's Peptone by HCN in the Presence of Acid and Alkali.*

ACID OR ALKALI	TRYPTOPHANE TEST	
	Without HCN	With HCN (.15 per cent)
None.....	None	Strong
0.1 cc. $\frac{N}{10}$ HCl.....	None	Strong
0.6 cc. $\frac{N}{10}$ HCl.....	None	Strong
0.1 cc. $\frac{N}{1}$ HCl .....	None	Marked
0.6 cc. $\frac{N}{1}$ HCl .....	None	Very faint
0.1 cc. $\frac{N}{10}$ Na <sub>2</sub> CO <sub>3</sub> .....	None	Strong
0.6 cc. $\frac{N}{10}$ Na <sub>2</sub> CO <sub>3</sub> .....	None	Marked
0.1 cc. $\frac{N}{1}$ Na <sub>2</sub> CO <sub>3</sub> .....	None	Distinct
0.6 cc. $\frac{N}{1}$ Na <sub>2</sub> CO <sub>3</sub> .....	None	Faint

*The Acceleration of the Digestion of Egg-white by HCN in the Presence of Acid and Alkali.*

(15 cc. egg-white + 5 cc. papain = 0.383 gram protein.)

ACID OR ALKALI	PROTEIN DIGESTED	
	Without HCN	With HCN (.15 per cent)
None.....	gram 0.040	gram 0.276
0.5 cc. $\frac{N}{10}$ HCl.....	0.042	0.277
2.5 cc. $\frac{N}{10}$ HCl.....	0.073	0.055
1.0 cc. $\frac{N}{1}$ HCl .....	0.022	0.013
2.5 cc. $\frac{N}{1}$ HCl .....	0.007	0.008
0.5 cc. $\frac{N}{10}$ Na <sub>2</sub> CO <sub>3</sub> .....	0.024	0.275
2.5 cc. $\frac{N}{10}$ Na <sub>2</sub> CO <sub>3</sub> .....	0.022	0.263
1.0 cc. $\frac{N}{1}$ Na <sub>2</sub> CO <sub>3</sub> .....	0.021	0.231
2.5 cc. $\frac{N}{1}$ Na <sub>2</sub> CO <sub>3</sub> .....	0.018	0.177

Hydrocyanic acid is effective even when considerable amounts of hydrochloric acid or sodium carbonate are present, whereas in no concentration did either this acid or alkali alone call forth a marked digestion of "peptone" or of egg-white. These experiments confirm the conclusion already reached, that *the acceleration of papain proteolysis by HCN is not due to a peculiarly favorable concentration of hydrogen ions.*

*Do Substances Related to HCN Produce a Similar Acceleration?*

Of substances more or less related to HCN, methyl cyanide and potassium sulphocyanide do not show a similar accelerating power over proteolysis by papain. That potassium cyanide does resemble HCN is to be expected from the preceding experiment. These compounds were used in concentrations approximately equivalent to that of the HCN in all previous experiments.

Fifteen cc. egg-white solution; 5 cc. 1 per cent papain solution; salts as indicated in the table; total volume 30 cc.; toluene; digested at 40° for 17 hours.

*The Effect on Papain Proteolysis of Substances related to HCN.*

(15 cc. egg + 5 cc. papain = .399 gram.)

REAGENT	PROTEIN DIGESTED
	<i>gram</i>
Hydrocyanic acid, 0.15 per cent.....	0.290
Potassium cyanide, 0.36 per cent.....	0.160
Methyl cyanide, 0.2 per cent.....	0.049
Potassium sulphocyanide, 0.54 per cent....	0.035

*Does HCN Destroy Inhibitory Substances in the Papain?*

In view of the well known inhibition of catalase by HCN,<sup>1</sup> it was thought that the acceleration of papain proteolysis by this substance might be due to an indirect action—the inhibition or destruction of a catalase, peroxidase or peroxide in the papain powder, which ordinarily exerted an inhibitory influence over the proteolytic enzyme. No evidence was obtained of the liberation of oxygen from hydrogen peroxide or of the bluing of guaiaconic acid either by papain alone or by papain and hydrogen peroxide. A wide range of concentrations was used, with comparative tests on carrot juice for controls. Apparently the papain powder contained neither catalase, peroxide or peroxidase in any conspicuous amount.

The possibility still remained that HCN destroys some other inhibiting agent in the papain. If this is the explanation of its

<sup>1</sup> Cf. Schönbein: *Journ. f. prakt. Chem.*, cv, p. 202, 1868.



action, removal of the HCN from papain which has stood with it for some time, either by a current of air or by dialysis, should leave an active enzyme.

To test this point, 75 cc. of a 1 per cent aqueous<sup>1</sup> extract of papain were allowed to stand with 18.7 cc. of 1.8 per cent HCN<sup>2</sup> and toluene in the cold for 7 hours. The HCN was removed from a portion of this mixture by bubbling air through it for 3 hours. From a second portion the HCN was removed by dialysis for 18 hours against running water with toluene as antiseptic. The third portion was kept for comparison. To show that the enzyme was not destroyed by the treatment, digestions were made not only with the dialyzed and aerated material, but with these solutions after a second addition of HCN.

Three cc. Witte's peptone solution; 3.7 cc. of the papain solutions, treated as described above and indicated in the table (equivalent to 3 cc. of the original 1 per cent solution); HCN as indicated in the table; toluene; digested at 40° for 18 hours.

*Does HCN Produce a Permanent Change in the Papain Solution?*

TRYPTOPHANE TEST		
TREATMENT OF PAPAÏN	HCN restored (.15 per cent)	
Papain has stood with HCN for 28 hours.....	Strong	.....
Same papain mixture dialyzed free from HCN .....	None	Strong
Same papain mixture aerated free from HCN.....	None	Strong

Fifteen cc. egg-white solution; 12.5 cc. of the papain solutions described in the previous experiment (equivalent to 10 cc. of the original 1 per cent solution); HCN as indicated in the table; toluene; total volume 30 cc.; digested at 40° for 5 hours.

<sup>1</sup> An aqueous rather than a saline extract of papain was used, to avoid change of volume when the papain was dialyzed.

<sup>2</sup> This proportion of HCN to papain is the same *by weight* as that used in most of the experiments on egg-white. It is slightly greater than that in the experiments on peptone.

*Does HCN Produce a Permanent Change in the Papaïn Solution:*  
(15 cc. egg + 12.5 cc. papaïn = 0.405 gram protein.)

TREATMENT OF PAPAÏN	PROTEIN DIGESTED	
		HCN restored(.15 per
	gram	gram
Papaïn had stood with HCN* for for 28 hours.....	0.269	
Same papaïn mixture dialyzed free from HCN.....	0.014	0.208†
Same papaïn mixture aërated free from HCN.....	0.011	0.201

\*The concentration of the HCN in this mixture, after the addition of the egg so was comparable to that in the other tests containing HCN (0.15 per cent).  
†That the aërated and dialyzed solutions show some deterioration of the enzyme surprising when it is remembered that papaïn solutions deteriorate rather rapidly on standing, cf. p. 201.

Inasmuch as the removal of HCN from a papaïn solution w  
has stood with it leaves the enzyme as inactive as it was origin  
*the acceleration of proteolysis by HCN cannot be attributed t*  
*destruction of an inhibiting substance in the papaïn powder.*  
experiment does not exclude the rather remote possibility  
the HCN inhibits rather than destroys an inhibiting substan

*Does HCN Destroy Inhibitory Substances in the Substra*

It is well known that certain uncoagulated protein mixt  
especially serum, are very resistant to digestion, and that  
reistance disappears when they are coagulated. The phenom  
has been explained by some as due to antienzymes associated  
these proteins; by others, to peculiarities in the proteins t  
selves. If only the raw egg digestions were considered, one v  
be tempted to attribute the acceleration of papaïn proteolys  
HCN to the destruction of an antienzyme or to a denaturing  
on the protein comparable to heat coagulation. This view o  
matter is contradicted by the fact that a striking example c  
celeration by HCN is seen not only in the digestion of native pr  
but in that of "peptone." That HCN does not *permanently* ch

egg-white in the direction of making it more easily digestible by papain is demonstrated by an experiment in which egg-white that had stood for some time with HCN was dialyzed free from this substance.

Thirty cc. of egg-white solution which had stood for 5 hours with 5 cc. of 1.8 percent HCN were dialyzed against running water with toluene for antiseptic for 24 hours. The dialysate was made up to 50 cc., and 25 cc. (corresponding to 15 cc. of the original egg-white solution) were digested with 1 cc. of 1 per cent papain solution, with toluene as antiseptic, for 17 hours at 40°. A comparative test was made with 15 cc. of the original egg-white solution, 1 cc. of 1 per cent papain solution, and HCN (concentration = 0.15 per cent). Total volume in both cases was 30 cc.

The mixture of egg-white and papain contained 0.422 gram of protein precipitable by trichloroacetic acid. Of this, 0.222 gm. were digested in the presence of HCN by the amount of papain used. Of the egg which had been treated with HCN and then dialyzed free from it, only 0.077 gm. were digested.

*The action of HCN is apparently not due to the destruction of an inhibitory substance in the protein mixture or to a permanent chemical change in the protein.*

### *Does HCN Activate a Papain Zymogen?*

There remains for consideration the question whether HCN does not activate a papain zymogen as calcium salts activate trypsinogen. The answer is found in the experiments (cf. p. 195) in which papain which stood with HCN for some time was dialyzed. The change from a zymogen to an enzyme is an irreversible process. If HCN is an activator, removal of this substance from papain previously treated with it should leave an active enzyme. The experiments already cited show conclusively that this is not the case, although the dialyzed papain becomes active if HCN is added anew. *The acceleration by HCN cannot be attributed to the activation of a zymogen.*

### PHENOMENA OF ENZYME DETERIORATION AND THE RÔLE OF TEMPERATURE.

The recent literature of papain contains contributions which indicate two striking peculiarities in the behavior of this enzyme toward certain proteins at low and high temperatures. The phe-

nomena, first described by Delezenne, Mouton, and Pozerski<sup>1</sup>, 1906, have recently been discussed at greater length by Pozerski.<sup>2</sup>

According to these authors, papain digests the proteins of egg white and serum so rapidly at high temperatures (80° or 90°) that if a suitable mixture of enzyme and uncoagulated protein faintly acidified, is heated quickly to boiling over a free flame, scarcely any coagulum is obtained.<sup>3</sup> At 40°, the temperature usually considered the most favorable for digestion, the proteins in question are not measurably hydrolyzed by papain during short periods. Furthermore, these proteins are said to rapidly render the enzyme inactive at 40°, so that subsequent digestion when the mixture of protein and enzyme is heated to boiling is less and less marked, depending on the length of contact at the lower temperature.

These apparent anomalies the authors interpret as depending upon peculiarities both in the enzyme and in the substrata. The rapidity of digestion at high temperatures—to consider that first—is the result of a balance between a number of factors. Papain, they assume, differs from pepsin and trypsin in being less quickly destroyed with rising temperature; and, during the period of survival, it is more and more active the higher the temperature. When they are denatured by heat, the proteins of egg-white and serum lose the resistance to digestion which they show in their native condition. At and above the coagulation temperature, the conditions with this enzyme are peculiarly favorable, for short periods of time, to a rapid digestion. The total period of activity of the enzyme is shorter, the higher the temperature.

The explanation of the destruction of papain by uncoagulated egg-white is less satisfactory. Jonescu<sup>4</sup> and Sachs,<sup>5</sup> who independently verified the main facts as stated by Delezenne, Mouton and Pozerski almost immediately after their publication, have inter-

<sup>1</sup> Delezenne, Mouton, and Pozerski: *Compt. rend. soc. de biol.*, lx, p. 68, 309, 1906.

<sup>2</sup> Pozerski: *Ann. de l'Inst. Pasteur*, xxiii, p. 205, 321, 1909.

<sup>3</sup> This recalls forcibly the accounts in the early literature on the use of the pawpaw milk by the natives in the tropics. Thus Griffith Hughes (*The Natural History of Barbadoes*, 1750) says, "This juice is of so penetrating a nature, that if the unripe fruit when unpeeled is boiled with the toughest old salt meat, it will soon make it soft and tender."

<sup>4</sup> Jonescu: *Biochem. Zeitschr.*, ii, p. 177, 1907.

<sup>5</sup> Sachs: *Zeitschr. f. physiol. Chem.*, li, p. 488, 1907.

ed this phenomenon in terms of the Ehrlich side chain theory being due to the fixation of the enzyme by the receptors of uncoagulated protein. This explanation can hardly be said to rest upon a firm experimental basis. Sachs has, however, made several definite contributions to the study of the mechanism of autoinactivation. According to him, it is not accounted for by the spontaneous deterioration of the papain on standing in solution, the action of the alkali of the egg, although it is prevented by small amounts of hydrochloric acid.

Gerber<sup>1</sup> has recently reported peculiarities in the rennetic activity of papain which closely parallel those described for raw egg albumen. Milk is clotted very rapidly by papain at high temperatures. Gerber asserts, furthermore, that papain is rapidly rendered inactive by the lactalbumin and serum globulin of the milk, a fact which he supports with two facts. Milk previously heated above the coagulation temperature of these proteins is clotted more rapidly than raw milk. If the amount of papain is sufficient to clot the milk within a few minutes, it does not clot at all.

In view of the prevailing idea that the optimum temperature for the activity of enzymes is in the neighborhood of 40° the observations of Delezenne and his co-workers seem revolutionary. As Gerber<sup>2</sup> has pointed out, however, the idea that the most favorable temperature for the activity of papain is higher than for pepsin or trypsin is not entirely new. Both this author and Pozerski overlooked the observation of Chittenden<sup>3</sup> that papain in solution digests more meat protein at 70° than at any lower temperature.

Chittenden<sup>4</sup> has suggested that the rapidity of digestion by papain at high temperatures provides a method for testing for this enzyme. It is already clear, however, that papain does not stand in a class by itself in this respect. In 1892 Chittenden<sup>5</sup> reported that the juice of pineapple (*Ananas sativus*) is most active on

Gerber: *Compt. rend. soc. de biol.*, lxxvii, p. 332, 1909.

Sachs: *loc. cit.*

Chittenden: *Trans. Conn. Acad. of Arts and Sci.*, ix, p. 311, 1892.

Abderhalden's *Handbuch der Biochemischen Arbeitsmethoden*, iii, 1910.

Chittenden: *Trans. Conn. Acad. of Arts and Sci.*, viii, p. 293, 1892.

coagulated egg albumin at 50°-60°. and Pozerski<sup>1</sup> has found that under the more favorable conditions established by his procedure this enzyme digests egg-white rapidly at 80°-90°.<sup>2</sup> The rennins of many plants, other than the pawpaw, are, according to Gerber, active at relatively high temperatures. The most striking example is the rennin of *Atropa belladonna*<sup>3</sup> which is most active at 90° and will even clot *boiling* milk provided enough enzyme is added to act within a short time. There is, therefore, every reason to believe that further study will disclose still other ferments in which the acceleration of the rate of action by heat more than compensates for the rate of destruction of the enzyme, so that for short periods, under favorable conditions, the rate of digestion may be high.

After all, it is less surprising that vegetable enzymes should show considerable individuality than that they should all be most active at animal body temperature.<sup>4</sup> Whether the enzymes are protein in their nature or not, they are closely associated with protein, and it may well be that the relative resistance of ferments of plant origin to high temperatures is connected with the comparative stability of the plant proteins to heat.<sup>5</sup>

The rapid destruction of papain by egg-white and serum reported by Pozerski seems to form an exception to the current idea that enzymes are protected from deterioration by their respective substrata.<sup>6</sup> The most obvious explanation of this anomaly, namely, that the apparent destruction of papain by these proteins is really due to a spontaneous degeneration of the enzyme in solution, has been tested by Sachs, as stated above. Pozerski himself gives no data on this point, but merely says that "it is easily shown that the spontaneous degeneration of papain on standing alone is far from sufficient to explain the observed results."<sup>7</sup>

<sup>1</sup> Pozerski: *loc. cit.*, p. 341.

<sup>2</sup> He failed to demonstrate the destruction of bromelin by egg-white at low temperatures which was such a striking feature of his work on papain.

<sup>3</sup> Gerber: *Compt. rend. soc. de biol.*, lxxvii, p. 318, 1909.

<sup>4</sup> Recent studies of animal amylases (Slosse and Limbosch: *Arch. internat. physiol.*, vi, p. 365, 1908) and of pepsin and trypsin (Roeder: *Biochem. Zeitschr.*, xxiv, p. 496, 1910) indicate that the optimum temperature for the activity of these enzymes is considerable above body temperature.

<sup>5</sup> Cf. Osborne: *The Vegetable Proteins*, p. 44, 1909.

<sup>6</sup> Cf. Samuely in Oppenheimer's *Handbuch der Biochemie*, i, p. 513, 1909.

<sup>7</sup> Pozerski: *loc. cit.*, p. 329.

From a repetition of the experiments of Pozerski, we were convinced of the validity of his observations as to the rapidity of digestion by papain at 80° or 90°; but we obtained *no evidence that papain solutions deteriorate more rapidly when they are mixed with white of egg than when they stand alone for the same length of time. On the contrary, the protein exerts a slight protective action over the enzymes.* Experiments were made with six different samples of papain, three of which were from Merck and one from Johnson and Johnson. A fifth sample consisting of sun-dried latex is described in detail elsewhere (cf. p. 183). Lastly experiments were made with perfectly fresh pawpaw milk (cf. p 206.)

Inasmuch as the results obtained with these various preparations were entirely similar, only a few typical protocols need be given.

*A Comparison of the Deterioration of Papain on standing alone and with Egg-white.*

The egg-white for use in these experiments was beaten slightly, diluted with 3 volumes of 1 per cent sodium chloride solution and filtered. The papain solution, freshly prepared for each experiment was a 1 per cent or 2 per cent extract in 1 per cent sodium chloride solution. The amount of papain used was varied, depending upon the conditions of the experiment. Digestions were made in stoppered Erlenmeyer flasks with toluene for antiseptic. The papain for the control experiments was always measured into a clean dry flask at the same time that the papain and egg for the 40° digestions were mixed, and after the addition of toluene put in the thermostat at 40°. After the length of time specified in the tables, egg was added to this papain and the mixture digested at the high temperature simultaneously with the mixture which had stood at 40° for the same time. Various procedures, indicated in individual experiments, were employed to bring the mixtures to the boiling point. The coagula were filtered on dried tared filters, washed free from acid and chlorides and dried to constant weight. The amount of protein in the egg solution coagulable by heat was determined in a blank.

I.

Fifteen cc. egg white solution; 10 cc. 1 per cent papain solution;<sup>1</sup> toluene. In one series, the papain stood with toluene at 40° for the length of time specified in the table before it was mixed with the egg; in the second series, the papain and egg stood together for the same lengths of time at 40°. To

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<sup>1</sup> In this experiment and the three which follow the papain described on p. 183 was used.

bring the mixtures to boiling they were put for 15 minutes in an 80° bath then for 5 minutes in a 100° bath. They were all acidified before heating 1 cc.  $\frac{N}{2}$  acetic acid.  
(15 cc. egg solution = 0.380 gram protein.)

TIME AT 40°	PROTEIN DIGESTED	
	Papain and Egg Stood Separately	Papain and Egg Stood Together
	gram	gram
At once.....	.....	0.161
15 minute .....	0.154	0.157
30 minutes.....	0.147	0.157
1 hour.....	0.135	0.148
4 hours.....	0.075	0.131
24 hours.....	0.032	0.106

II.

This test differs from the last in that the mixtures were not acidified just before putting them in the 100° bath. Digestion is so much more rapid at 80° at the natural alkalinity of the egg than in acid solution<sup>1</sup> that no less papain was used (1 cc. of a 1 per cent solution). The volume was made up to 25 cc. with 1 per cent sodium chloride solution.  
(15 cc. egg solution = 0.380 gram protein.)

TIME AT 40°	PROTEIN DIGESTED	
	Papain and Egg Stood Separately	Papain and Egg Stood Together
	gram	gram
At once.....	.....	0.204
15 minutes.....	0.188	0.199
30 minutes.....	0.178	0.201
1 hour.....	0.147	0.194
4 hours.....	0.149	0.162
24 hours.....	0.145	0.154

III.

This experiment is similar to the two already cited, except in the method of heating. The mixtures were put in a 100° bath for 5 minutes<sup>2</sup> with

<sup>1</sup> Cf. Sachs: *loc. cit.*  
<sup>2</sup> The enzyme is almost completely destroyed during 5 minutes at 100°



any previous heating at 80°. They were then acidified with 1 cc.  $\frac{N}{2}$  acetic acid and heated for 5 minutes longer. 10 cc. of a 2 per cent solution of papain was used.

(15 cc. egg solution = 0.380 gram protein.)

TIME AT 40°	PROTEIN DIGESTED	
	Papain and Egg Stood Separately	Papain and Egg Stood Together
	gram	gram
At once.....	.....	0.264
15 minutes.....	0.261	0.258
30 minutes.....	0.254	0.256
1 hour.....	0.251	.....
4 hours.....	0.205	0.227
24 hours.....	0.129	0.194

DETERIORATION IN THE PRESENCE AND ABSENCE OF ANTI-SEPTICS. In the experiments just cited toluene was used as anti-septic, whereas in most of those reported by Pozerski, Sachs and Jonescu respectively no antiseptic was used. The following table indicates that the differences between our results and theirs is not attributable to the antiseptic.

Fifteen cc. egg-white solution; 10 cc. 2 per cent papain solution; anti-septics as indicated in the table. In one series the papain stood alone or with the antiseptic for 20 hours at 40° before it was mixed with the egg; in a second series the papain and egg stood together under the same conditions; in a third series the digestive power was tested at once. Digestions were made in a 100° bath as in the last experiment.

(Fifteen cc. egg solution=0.374 gram protein.)

ANTISEPTIC	PROTEIN DIGESTED		
	At Once	Papain and Egg Stood Separately	Papain and Egg Stood Together
	gram	gram	gram
Toluene.....	0.267	0.211	0.230
Chloroform, .05 per cent.....	0.261	0.203	0.201
Sodium fluoride, .05 per cent.....	0.271	0.228	0.248
None.....	0.270	0.230	0.258

These experiments show quite conclusively that *the spontaneous deterioration of papain on standing in solution more than accounts for the deterioration observed when it stands with white of egg. The protein, if anything, protects the enzyme from loss of activity.* This protective action was equally marked in an experiment with the fresh milky juice from pawpaw. (cf. p. 206).

*The Digestion of Vegetable Proteins by Papain at High Temperatures.*

Preliminary experiments on the digestion of excelsin and edestin by papain indicate that the contrast between the rate of digestion at 40° and at 80° is hardly less striking with typical plant proteins than with egg albumin.

A suitable solution of edestin was prepared by shaking 4 grams of the protein with 150 cc. of 10 per cent sodium chloride solution and filtering off an undissolved residue. The excelsin solution was obtained by titrating a suspension of 5.6 grams of excelsin in 140 cc. of water with  $\frac{N}{2}$  NaOH to the point of faint alkalinity to phenolphthalein and filtering off an undissolved residue. For the tests, 10 cc. of these protein solutions were used with 10 cc. of 1 per cent papain solution. The undigested protein was determined by precipitating with an equal volume of hot trichloroacetic acid. (Cf. p. 186)<sup>1</sup>.

10 cc. edestin solution = 0.21 gram protein.

10 cc. excelsin solution = 0.27 gram protein.

TEMPERATURE AND TIME	EDESTIN DIGESTED	EXCELSIN DIGESTED
	gram	gram
40°, 17 hours.....	0.07	0.07
80°, 15 minutes.....	0.14	0.20

*The Formation of Amino-acids by Papain at High Temperatures.*

Previous investigators of the phenomena of digestion by papain at high temperatures have reported that the enzyme effects a true

<sup>1</sup> As has already been stated, trichloroacetic acid does not appear to give quantitative precipitation of these proteins under the conditions of the experiment. Inasmuch as the precipitations were all made under strictly comparable conditions, it is fair to assume, however, that the experiments give an approximately true idea of the relative amounts of digestion.

digestion with the formation of peptone<sup>1</sup> but they have found no evidence of the liberation of amino-acids. Our own experiments indicate that if the conditions are made especially favorable for rapid digestion, by the use of a comparatively high concentration of enzyme and by the addition of hydrocyanic acid to the digestion mixture, the formation of amino-acids at high temperatures can easily be demonstrated.

Five cc. of 5 per cent Witte's peptone digested with 5 cc. 5 per cent papain solution and 0.15 per cent HCN at 80° for 15 minutes gave a strong tryptophane test with bromine water.

#### CONFIRMATORY EXPERIMENTS WITH FRESH LATEX.

The fresh latex of the pawpaw<sup>2</sup> shows the same general peculiarities as the dried material.

A drop of the milky juice about the size of a wheat grain completely liquefied a 5 per cent gelatin plate (0.1 per cent thymol) in 3 hours at room temperature. The same amount clotted 5 cc. of milk instantly at 40° and dissolved a large flock of fibrin suspended in 5 cc. of 0.1 per cent thymol water in 5 hours at 40°. Sharply contrasted with this was its relative inactivity on peptone. In a mixture consisting of 5 cc. of a 2 per cent solution of Witte's peptone, the same amount of latex used in the other experiments, and toluene for antiseptic, only the merest trace of tryptophane was detected after 3 days digestion at 40°. In a comparable digest to which HCN was added (0.15 per cent) a strong reaction for tryptophane was obtained after 17 hours.

These experiments indicate that even with the fresh latex, which is otherwise extremely active, the formation of amino-acids is very slow unless HCN is present.

<sup>1</sup> Cf. Pozerski: *loc. cit.*

<sup>2</sup> The fresh latex was obtained from green pawpaws (*Carica papaya*) imported from Jamaica. Small droplets of latex, which clot almost immediately, ooze out when the skin is cut. This material is, judging from Kilmer's description (*Amer. Journ. of Pharm.*, lxxiii, p. 272, 336 and 383, 1901) comparable with the commercial product which is obtained by making incisions in the green fruit while it is still on the tree. According to him, the latex spurts out with considerable force when a cut is made, but the flow is soon stopped by spontaneous clotting. Specimens of ripe fruit give no latex when the skin is cut, and the pulp shows very little proteolytic activity.

A typical quantitative experiment with egg-white will serve to show that the accelerating action of HCN is not limited to the “ereptic” activity of the latex.

One and one-half grams of latex were diluted with 50 cc. of 1 per cent sodium chloride solution and the mixture filtered. The experiment was similar in plan to that described in detail on p.186 except that 10 cc. of the enzyme solution were used. The egg white solution contained 0.422 gram of protein coagulable by trichloracetic acid. After 17 hours digestion, with toluene for antiseptic, 0.034 gram were digested; with toluene and HCN, 0.399 gram.

The fresh latex, like the dried samples, digests egg-white rapidly at 80°–100°; deteriorates spontaneously on standing in solution; and is protected from deterioration by egg-white.

One and one-half grams of fresh pawpaw latex were diluted with 60 cc. of 1 per cent saline and filtered. 10 cc. of this solution were used with 10 cc. of egg solution and the heating conducted as in experiment II, p. 202.

(15 cc. egg solution = 0.422 gram protein.)

TIME AT 40°	PROTEIN DIGESTED	
	Papain and Egg Stood Separately	Papain and Egg Stood Together
	gram	gram
At once.....	.....	0.305
17 hours.....	0.169	0.288

ANTIENZYMES: THE INFLUENCE OF ASCARIS EXTRACTS ON PAPAÏN PROTEOLYSIS.

The press juice from certain intestinal worms was shown by Weinland<sup>1</sup> to exert a marked inhibitory power over the digestion of fibrin by pepsin and by trypsin. This activity he attributed to an antienzyme (or antienzymes) which serves to protect the worms from digestion in the alimentary canal. It is a matter of some inter-

<sup>1</sup> Weinland: *Zeitschr. f. Biol.*, xliv, p. 1, 1903. Cf. also, Hamill: *Journ. of Physiol.*, xxxiii, p. 479, 1905–6; and Fetterolf: *Univ. of Penn. Med. Bull.*, xx, p. 94, 1907.

est, in relation to the question how closely the proteolytic enzyme of plants resemble those in animals, to know whether these worm extracts inhibit proteolysis by ferments of plant origin.

**THE PREPARATION OF AN ANTIENTZYME SOLUTION FROM *ASCARIS*.** Preliminary experiments showed that the method formerly used for obtaining an antientzyme solution from *Ascaris*, namely, that of grinding and pressing the entire worm, leads to false conclusions when certain methods for detecting digestion are used, owing to the fact that such a press juice contains a large amount of erepsin. This enzyme<sup>1</sup> was found to be localized in the alimentary canal or its contents. The error arising from its presence in the antientzyme solution could, therefore, be avoided by dissecting out the digestive tract before grinding the worm. A typical preparation of an antientzyme extract is here given in detail.

One hundred grams of *Ascaris* (from pigs) were washed thoroughly. The entire alimentary canal and the genital organs were carefully dissected out. The body walls of the worms, after they had been elaborately washed with water to remove traces of erepsin, were ground thoroughly<sup>2</sup> with an equal volume of quartz sand, and extracted over night with 200 cc. of 1 per cent NaCl solution, with toluene for antiseptic. The press juice was very milky in appearance and could not be filtered clear. After it had stood for 24 hours in the cold room, the suspended material had flocked together and a filtrate which was only slightly opalescent could be obtained. This solution was faintly acid to litmus and gave considerable coagulum when heated. The freshly prepared extracts showed a trace of ereptic activity<sup>3</sup> which disappeared in a day or two.

*The extract of Ascaris prepared in this way was strongly antipeptic and antitryptic, but not the slightest evidence was obtained that*

<sup>1</sup> This erepsin corresponds in properties, so far as they were investigated, with that in the mucosa of the pig's intestine. It digests Witte's peptone to amino-acids, liquefies gelatin, clots milk and subsequently dissolves the clot, but does not attack fibrin. Abderhalden and Heise (*Zeitschr. f. physiol. Chem.*, lxii, p. 136, 1909) found that the isolated intestine of *Ascaris canis* split tyrosine from peptone "Roche." Fermi and Repetto (*Centralbl. f. Bakt.*, xxxi, p. 403, 1902) reported that a large number of intestinal parasites do not liquefy gelatin. According to Kobert (*Arch. f. d. ges. Physiol.*, xcix, p. 121, 1903) *Ascaris* extracts digest fibrin but do not clot milk.

<sup>2</sup> As Weinland (*loc. cit.*) has pointed out, the success of the experiment depends largely on how thoroughly the material is ground.

<sup>3</sup> 2 cc. digested with 2 cc. of 5 per cent Witte's peptone solution over night at 40° gave a perceptible tryptophane test.

it inhibited proteolysis by papaïn.<sup>1</sup> This striking difference is illustrated by the following typical protocols showing the effect of *Ascaris* extract on the digestion of gelatin and of fibrin and on the clotting of milk by trypsin and by papaïn respectively.

**THE INFLUENCE OF *Ascaris* EXTRACTS ON THE LIQUEFACTION OF GELATIN BY TRYPSIN.** The inhibiting effect of the *Ascaris* extract on the digestion of gelatin by trypsin was determined by comparing the amount of enzyme necessary, in the presence and in the absence of the *Ascaris* extract, to hydrolyze gelatin under definite conditions to a point at which the solution does not solidify on cooling.

To tubes containing 1 cc. of "melted" 5 per cent gelatin solution (containing 0.5 per cent thymol) were added: to one series, 0.5 cc. water; to a second series, 0.5 cc. *Ascaris* extract. A 0.1 per cent solution of trypsin (Fairbank's) in  $\frac{N}{10}$  sodium carbonate solution was added in the amounts indicated in the following table, and  $\frac{N}{10}$  sodium carbonate solution to make a total volume of 2 cc. The mixtures were placed at 40° for 15 minutes and examined for digestion by chilling in cold water. They were returned to the thermostat and examined after 48 hours.

*The Influence of Ascaris Extracts on the Liquefaction of Gelatin by*

TRYPSIN SOLUTION	CONDITION OF GELATIN			
	AFTER 15 MIN.		AFTER 48 HOURS	
	Without <i>Ascaris</i>	With <i>Ascaris</i>	Without <i>Ascaris</i>	With <i>Ascaris</i>
cc.				
0.05	Solid	Solid	Liquid	S
0.10	Liquid	Solid	Liquid	S
0.15	Liquid	Solid	Liquid	S
0.20	Liquid	Solid	Liquid	S
0.25	Liquid	Solid	Liquid	S
0.30	Liquid	Solid	Liquid	S
0.35	Liquid	Solid	Liquid	L
0.40	Liquid	Solid	Liquid	L

<sup>1</sup> The older literature on papaïn has a statement that this enzyme dissolves *Ascaris* (Wurtz and Bouchut: *Compt. rend. de l'acad. des Sci.* p. 426, 1879). Herzog (*Zeitschr. f. physiol. Chem.*, xxxix, p. 305, 1905) used the antienzyme of *Ascaris* to study the question whether "proteolysis" is to be attributed to proteolytic enzymes, reported that the press juice of *Ascaris* exerts the same inhibition over papaïn as ovalbumin. His data do not seem to justify this conclusion.

THE INFLUENCE OF *Ascaris* EXTRACTS ON THE LIQUEFACTION OF GELATIN PAPAÏN. An experiment with papaïn<sup>1</sup> planned on similar lines showed sign of inhibition of proteolysis by the *Ascaris* extract, whether the medium was  $\frac{N}{10}$  with sodium carbonate, as in the experiment with trypsin, practically neutral.<sup>2</sup> For the alkaline tests, 0.1 per cent papaïn was dissolved in  $\frac{N}{10}$  sodium carbonate and the volumes were made up with  $\frac{N}{10}$  sodium carbonate. For the neutral tests, 0.1 per cent papaïn in water was used and volumes were made up with water. Liquefaction was considerably retarded by alkali in the concentration used. The table records the condition of the gelatin, in the alkaline digest, at the end of an hour; in the neutral, at the end of 15 minutes.

*Influence of Ascaris Extracts on the Liquefaction of Gelatin by Papaïn.*

PAPAÏN SOLUTION	CONDITION OF GELATIN			
	ALKALINE SOLUTION		NEUTRAL SOLUTION	
	Without <i>Ascaris</i>	With <i>Ascaris</i>	Without <i>Ascaris</i>	With <i>Ascaris</i>
cc.				
0.05	Solid	Solid	Solid	Solid
0.10	Solid	Solid	Solid	Solid
0.15	Solid	Solid	Solid	Solid
0.20	Solid	Solid	Solid	Solid
0.25	Solid	Solid	Solid	Solid
0.30	Solid	Solid	Solid	Solid
0.35	Solid	Solid	Solid	Solid
0.40	Liquid	Liquid	Solid	Liquid
0.45	Liquid	Liquid	Solid	Liquid
0.50	Liquid	Liquid	Liquid	Liquid

THE INFLUENCE OF *Ascaris* EXTRACTS ON THE SOLUTION OF FIBRIN BY TRYPSIN. The quantities of 0.1 per cent trypsin in  $\frac{N}{10}$  sodium carbonate solution indicated in the table, were diluted, for one series with 2 cc. *Ascaris* extract, for the other with 2 cc. water. The volumes were made up to 3 cc. with  $\frac{N}{10}$  sodium carbonate solution, a flock of fibrin and a little toluene were added to each, and the mixtures were digested at 40° for 18 hours.

The papaïn used was the dried latex described on p. 183. In neutral solution digestion was even slightly accelerated—a result which is probably attributable to the salts in the *Ascaris* extract.

*The Influence of Ascaris Extracts on the Solution of Fibrin by Trypsin.*

TRYPSIN SOLUTION	CONDITION OF FIBRIN	
	Without <i>Ascaris</i>	With <i>Ascaris</i> <sup>*</sup>
cc.		
0.2	Well disintegrated	Firm
0.4	Almost dissolved	Firm
0.6	Almost dissolved	Firm
0.8	Dissolved	Firm

<sup>\*</sup> There was no sign of digestion in the tests containing the worm extract even after three days.

THE INFLUENCE OF *Ascaris* EXTRACTS ON THE SOLUTION OF FIBRIN BY PAPAÏN. A similar experiment with papain showed no inhibition of the solution of fibrin. A 1 per cent solution of papain was used and the volumes were made up to 5 cc. with water.

*The Influence of Ascaris Extracts on the Solution of Fibrin by Papain.*

PAPAÏN SOLUTION	CONDITION OF FIBRIN	
	Without <i>Ascaris</i>	With <i>Ascaris</i>
cc.		
1.0	Disintegrated	Disintegrated
2.0	Disintegrated	Disintegrated
3.0	Nearly dissolved	Nearly dissolved

THE INFLUENCE OF *Ascaris* EXTRACTS ON THE CLOTTING OF MILK BY TRYPSIN. A milk from which most of the fat had been extracted with chloroform was used as a substratum (cf. p. 188). To tubes containing 3 cc. of the prepared milk were added to one series 0.5 cc. water; to the second 0.5 cc. of the worm extract. A 0.2 per cent aqueous extract of trypsin was added in the amount indicated in the table and enough water to provide a final volume of 4 cc. The tests were put in a water bath at 40° and examined at intervals. The condition of the tubes at the end of half an hour is indicated in the table. A few drops of chloroform were added to each test.

<sup>1</sup> The familiar difficulties were encountered in finding conditions under which trypsin will produce a clot in milk. It is well to avoid any considerable dilution of the milk and dissolve the trypsin in water rather than alkali.



and digestion was continued for 18 hours. In that time the clots were almost completely dissolved and the solutions gave strong tryptophane reactions in the digests containing no *Ascaris* extract. In the tubes containing the antienzyme the milk was apparently unchanged.

*The Influence of Ascaris Extracts on the Clotting of Milk by Trypsin.*

TRYPSIN SOLUTION	CONDITION OF MILK	
	Without <i>Ascaris</i>	With <i>Ascaris</i>
cc.		
0.05	Unclothed	Unclothed
0.10	Unclothed	Unclothed
0.15	Unclothed	Unclothed
0.20	Unclothed	Unclothed
0.25	Clotted	Unclothed
0.30	Clotted	Unclothed
0.35	Clotted	Unclothed
0.40	Clotted	Unclothed

THE INFLUENCE OF *Ascaris* EXTRACTS ON THE CLOTTING OF MILK BY PAPAÏN. No such inhibiting effect of *Ascaris* extracts on the clotting of milk by papaïn was observed<sup>1</sup> in a comparable experiment in which a 0.2 per cent solution of papaïn in water was used instead of trypsin.

*The Influence of Ascaris Extracts on the Clotting of Milk by Papaïn.*

PAPAÏN SOLUTION	CONDITION OF MILK	
	Without <i>Ascaris</i>	With <i>Ascaris</i>
cc.		
0.10	Unclothed	Unclothed
0.15	Unclothed	Unclothed
0.20	Unclothed	Unclothed
0.25	Unclothed	Unclothed
0.30	Clotted	Clotted
0.35	Clotted	Clotted
0.40	Clotted	Clotted

<sup>1</sup>On the contrary, a slight acceleration was obtained comparable to that noted in the experiment on gelatin.

## SUMMARY.

The work of previous investigators has led to more or less confusing or contradictory statements regarding the enzymatic properties of the latex of the pawpaw (*Carica papaya*). Its proteolytic activities have not been entirely in accord with those described for the familiar proteases of animal origin. Vines has endeavored to demonstrate a plurality of enzymes in so-called papain, especially the existence of an erepsin. The unique behavior of HCN in contrast with other antiseptics (which are regarded as inhibitory) has been pointed out by him. Striking peculiarities in respect to the temperature optimum and to deterioration in the presence of uncoagulated protein have been indicated by others.

Our own experiments lead to the following conclusions. The digestion of Witte's peptone by papain in the presence of the common antiseptics, judged by the tryptophane test, is very slow; in the presence of HCN, hydrolysis is rapid. The striking difference between HCN and other investigated antiseptics is due to an *acceleration* of proteolysis by HCN. The accelerating effect is not limited to the hydrolysis of "peptone," but is also shown in the digestion of raw and coagulated egg-white, fibrin, edestin, and excelsin whether one take as the gauge of digestion the appearance of tryptophane, leucine and tyrosine, the conversion into products not precipitated by hot trichloroacetic acid or the rate of solution of insoluble protein. HCN also accelerates the clotting of milk and the liquefaction of gelatin. The bearing of this on the evidence for the existence of more than one proteolytic enzyme in papain is discussed.

Of various substances tried, hydrogen sulphide was the only one which produced an acceleration of digestion by papain comparable to that effected by HCN.

The activity of HCN cannot be attributed (a) to a peculiar favorable concentration of hydrogen ions; (b) to the destruction of an inhibiting substance in the papain; (c) to the destruction of an inhibiting substance in the substratum; (d) to a permanent denaturation of the substratum; or (e) to the activation of a papain zymogen. Pending further investigation, nothing remains but to compare the behavior of HCN with that of the so-called co-enzyme.

The rapid digestion of egg-white which papain effects when

mixtures of the enzyme and protein are heated to boiling has been confirmed; and it has been shown that typical plant proteins—excelsin and edestin—are digested with similar rapidity at 80°. Under the especially favorable conditions established by the presence of HCN, digestion at 80° proceeds to the amino-acid stage.

In regard to the marked deterioration which papain is said to undergo when it stands with uncoagulated egg-white, it appears that this cannot be considered a constant characteristic of the enzyme in question, inasmuch as it was found that in the six samples of papain which were studied the spontaneous deterioration of the enzyme on standing in solution in every case more than accounted for the loss of activity when it stood with the protein. Egg-white, if anything, protects papain from deterioration.

The fresh latex of the pawpaw resembles the dried material in its behavior toward antiseptics and temperature, and in the phenomena of deterioration.

Extracts of *Ascaris* which are strongly antiseptic and antitryptic exert no inhibition over papain proteolysis.

The behavior of papain toward *Ascaris* antienzymes, the acceleration phenomena induced by HCN, and the peculiar temperature relations, place papain in a different category from pepsin, trypsin and animal erepsin. Data already recorded or available from other plants<sup>1</sup> in respect to the HCN acceleration and other features place papain in contrast with other vegetable enzymes. The facts reported do not exclude the possibility of the simultaneous presence of more than one enzyme in the pawpaw latex. We prefer in this paper to place emphasis upon some of the unique aspects of enzyme behavior which have hitherto been overlooked, partly because attention has been directed so largely to the unorganized ferments of animal origin.

<sup>1</sup> Studies on the cabbage and pineapple are under way or about to be published; cf. Blood: *This Journal*, viii, p. 215, 1910, on the erepsin of cabbage.



## THE EREPSIN OF THE CABBAGE (*Brassica oleracea*).<sup>1</sup>

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Evidence was obtained by Vines<sup>2</sup> in 1903 that various parts of widely different species of plants contain enzymes which resemble the erepsin discovered shortly before by Cohnheim<sup>3</sup> in the intestinal mucosa of animals. In view of their probable importance in plant physiology and of the abundance of material which they offer for studies on the specificity of enzymes these plant erepsins have hardly received the attention they merit. Vines<sup>4</sup> has extended his studies chiefly in the direction of demonstrating that the so-called plant trypsins are really mixtures of a pepsin-like enzyme and an erepsin. Javillier<sup>5</sup> has studied the erepsin in *Lolium perenne* and Dean<sup>6</sup> that in the seeds of *Phaseolus vulgaris*.

The evidence adduced by Vines that plants contain erepsins was briefly this. Many plant juices and extracts<sup>7</sup> split off tryptophane rapidly from Witte's peptone, but entirely failed to hydrolyze fibrin as indicated by any apparent solution or by the formation of tryptophane. The amount of this amino-acid was judged by the

<sup>1</sup>An extract from the thesis presented by the author for the degree of Doctor of Philosophy, Yale University, June 1910.

<sup>2</sup>Vines: *Annals of Botany*, xvii, pp. 237, 597, 1903.

<sup>3</sup>Cohnheim: *Zeitschr. f. physiol. Chem.*, xxxiii, p. 451, 1901.

<sup>4</sup>Vines: *Annals of Botany*, xviii, p. 289, 1904; xix, pp. 149, 171, 1905; xx, p. 113, 1906; xxii, p. 103, 1908; xxiii, p. 1, 1909; xxiv, p. 213, 1910..

<sup>5</sup>Javillier: *Bull. soc. chim. de Paris*, 3<sup>e</sup> Ser., xxix, p. 693, 1903; *Compt. rend. acad. des sci.*, cxxxvi, p. 1013, 1903.

<sup>6</sup>Dean: *Botanical Gazette*, xxxix, p. 321, 1905; xl, p. 121, 1905.

<sup>7</sup>In some cases Vines used crushed tissue instead of expressed juice or extracts.

depth of color obtained when the digest was treated with chlorine water. Vines has reported very little work on other substrata. The few tests on raw egg-white were negative, and, what is surprising in view of the well-known fact that animal erepsin hydrolyzes casein, most of the ereptic solutions did not liberate any tryptophane from casein or from milk. Many of the plant juices contained more or less tryptophane originally; and in several the amount increased conspicuously when the juice was autoylzed, indicating that the erepsin was able to digest the proteins with which it was associated in the plant. Out of the large number of materials—leaves, stems, fruits, bulbs, tubers—studied by Vines, only the pulp of the apple and the orange and the sap of the white birch failed to show ereptic activity.

Quite independently of Vines, Javillier<sup>1</sup> came to the conclusion that in *Lolium perenne* the rennin which he was studying was associated with a casease, a gelatinase and an erepsin. The press juice from this plant clotted milk; converted casein and conglutin into products which turned black when treated with tyrosinase;<sup>2</sup> liquefied gelatin; and hydrolyzed peptone into products which no longer gave the biuret reaction. It did not digest either fibrin or coagulated white of egg. According to both Vines and Javillier, the plant erepsins digest native protein of plant origin although they are unable to hydrolyze animal proteins. Dean<sup>3</sup> found that this is not true of the erepsin in the seedlings of *Phaseolus vulgaris*. This enzyme did not digest phaseolin, excelsin or edestin. Both Javillier and Dean partially isolated their enzymes by the alcohol and the ammonium sulphate methods respectively.

In apparent contradiction of the conclusion reached by Vines that erepsins are widely distributed in plants stand the data of Fermi and Buscaglioni<sup>4</sup> on the power of plant tissues to liquefy gelatin. These authors failed to obtain liquefaction with approximately 50 per cent of the seeds, bulbs, tubers and roots which they studied; with 70 per cent of the fruits, and flowers; with 80 per cent

<sup>1</sup> Javillier: *loc. cit.*

<sup>2</sup> Cf. Harlay: *Journ. de pharm. et chim.*, 6<sup>e</sup> Ser., ix, p. 468, 1899.

<sup>3</sup> Dean: *loc. cit.*

<sup>4</sup> Fermi and Buscaglioni: *Centralbl. f. Bakt.*, 2 Abt., v, pp. 24, 63, 91, 125, and 145, 1899.

of the leaves and stems; and with 90 per cent of the plant juices. Furthermore, they recorded in many cases negative results with the very plants and parts of plants in which Vines subsequently found ereptic activity. For example, they failed to obtain liquefaction of gelatin with beet roots, onion bulbs, dahlia tubers, the epicarp of the orange, the pulp of the tomato and the grape, and the leaves of the geranium. Vines<sup>1</sup> attributed these differences to seasonal variations and to the different antiseptics used by him<sup>2</sup> (HCN or chloroform) and by Fermi (phenol). Another explanation than that given by Vines is suggested by the statements in the literature that human intestinal juice<sup>3</sup> and the erepsin of inactivated pancreatic juice<sup>4</sup> do not liquefy gelatin. Possibly plant erepsins are not able to hydrolyze this protein. The liquefaction of gelatin by plant tissues may be a function of their "trypsin" or it may be accomplished by a specific "gelatinase,"<sup>5</sup> although it would be hard to account, on teleological grounds, for the presence of such an enzyme in plants.

It has already been stated that Javillier speaks of a rennin and erepsin as associated in *Lolium perenne*. The question whether milk-clotting power and proteolytic activity are always "associated" in plants possesses considerable interest in connection with the well known theory developed by Pawlow and his pupils that the clotting of milk is not due to a specific enzyme, but is merely the first step in the hydrolysis of the casein by proteolytic ferments. Pawlow<sup>6</sup> has adduced as an argument in support of his theory the fact that plant extracts which are never called upon in nature to deal with milk, possess the milk-clotting power.

All the tryptic enzymes of plants which have been adequately

<sup>1</sup> Vines: *Annals of Botany*, xvii, p. 597, 1903.

<sup>2</sup> Vines claims that antiseptics have a much more specific influence on the rate of digestion than is commonly supposed. *Ibid.*, p. 610.

<sup>3</sup> Hamburger and Hekma: *Journ. de physiol. et de pathol.*, iv, p. 813, 1902.

<sup>4</sup> Bayliss and Starling: *Journ. of Physiol.*, xxx, p. 65, 1903-04. Dox (The Intracellular Enzymes of *Penicillium* and *Aspergillus*, *Bulletin 120*, Bureau of Animal Industry, United States Department of Agriculture, p. 45, 1910) has recently reported that the erepsins of the dog's intestine and of *Penicillium camemberti* respectively do liquefy gelatin.

<sup>5</sup> Cf. Pollak: Hofmeister's *Beiträge*, vi, p. 95, 1905.

<sup>6</sup> Pawlow and Parastchuk: *Zeitschr. f. physiol. Chem.*, xlii, p. 449, 1904.

studied do clot milk<sup>1</sup>; but trypsins have by no means been shown to have as wide a distribution in plants as have rennins.<sup>2</sup> Erepsins on the other hand, appear from Vines' work to be present in just those tissues—leaves and stems—in which rennins have been most frequently described. Unfortunately, studies on the rennetic and ereptic activity of the same plants and parts of plants, aside from that on *Lolium perenne*, have not been made. It is a curious circumstance that although Gerber,<sup>3</sup> who has recently conducted an extended study of plant rennins, has come to the same conclusion as did Vines in regard to erepsins—namely, that they are found almost universally throughout the plant kingdom—in no case did he and Vines study the same species.

The preceding review suggests a number of questions of interest chiefly in relation to the specificity of the proteolytic enzymes, which the data answer inadequately or not at all. Do the plant erepsins in general show rennetic activity? Do they differ from animal erepsins in not being able to digest casein, as Vines' experiments suggest? Do plant tissues show the power to clot milk independently of the power to digest its casein to amino-acids? Can the plant erepsins liquefy gelatin or is this power associated with "trypsin"? And lastly—a question which has great interest in relation to the function of these enzymes in the plant—can the plant erepsins hydrolyze the proteins with which they are associated?

These questions can be answered only by a careful investigation of a considerable number of individual erepsins. The experiments which follow were undertaken at the suggestion of Professor Lafayette B. Mendel. They describe the proteolytic activities of a typical enzyme of this class, that of the etiolated leaves of the

<sup>1</sup> Drosera, cf. Darwin's *Insectivorous Plants*, p. 113, 1875. Pawpaw, cf. Baginsky: *Zeitschr. f. physiol. Chem.*, vii, p. 209, 1883. Fig, cf. Hansen: *Sitzungsber. d. phys.-med. Ges. Würzburg*, 1884; see Maly's *Jahresb.*, xiv, p. 281, 1884. Seeds of *Ricinus communis*, cf. Green: *Proc. Roy. Soc. London*, xlviii, p. 391, 1890. Pineapple, cf. Chittenden: *Journ. of Physiol.*, xv, p. 249, 1894.

<sup>2</sup> For the earlier history of this subject, cf. Green: *Nature*, xxxviii, p. 274, 1888; *Annals of Botany*, vii, p. 88, 1893. See also Javillier: *Bull. soc. chim. de Paris*, 3<sup>e</sup> Ser., xxvii, p. 818, 1902; *Compt. rend. de l'acad. des sci.*, cxxxiv, p. 1373, 1902.

<sup>3</sup> Extended series of papers in *Compt. rend. de l'acad. des sci.* and *Compt. rend. soc. de biol.*, 1907-1910.



blage (*Brassica oleracea*). After a preliminary study of a few of the erepsin-yielding tissues cited by Vines, this material was chosen for the following reasons. 1. The expressed juice splits tryptophane from Witte's peptone with considerable rapidity. It does not itself give the tryptophane test and gives only the indole reaction after autolysis for several days.<sup>1</sup> This avoids the possible ambiguity in judging whether added protein is present. 3. It is one of the erepsins reported by Vines not to be alkali-stable.<sup>2</sup>

#### PREPARATION OF AN EREPTIC SOLUTION FROM CABBAGE.

In various methods for the partial purification of enzymes, ammonium sulphate precipitation, already used successfully by Chitenden<sup>3</sup> in separating the bromelin of pineapples, and Heim<sup>4</sup> in isolating the erepsin of the intestinal mucosa, and Dean,<sup>5</sup> in his work on *Phaseolus vulgaris*, seemed most promising. The only difficulty encountered was in filtering the juice obtained by grinding the cabbage. Sometimes the fine solid material flocked together if the juice was allowed to stand in a cold room over night; but with other samples a clear filtrate could be obtained only after the juice had been dialyzed some time. A description of the preparation which was used in the experiments recorded in this paper is here given in detail.

Cabbages used were firm small heads, the outer leaves of which contained a small amount of chlorophyll. Six of these gave, by grinding and filtering, 1800 cc. of juice which was very turbid and could not be filtered. This juice contained a large amount of sugar and was approximately neutralized to phenolphthalein. It was dialyzed in a parchment bag

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According to Vines considerable tryptophane is liberated when a watery extract of cabbage leaves is autolyzed. *Annals of Botany*, xvii, p. 249, 1903. (loc. cit.) verified Vines' observation on the power of cabbage juice to digest Witte's peptone. Fermi and Buscaglioni, (loc. cit.) note that this juice does not liquefy gelatin; their form of statement leaves some question as to what part of the plant they used.

Chitenden: *Trans. Conn. Acad. of Arts and Sci.*, viii, p. 281, 1892; *Physiol.*, xv., p. 251, 1894.

Heim: loc. cit.

Dean: loc. cit.

against running water, with toluene for antiseptic, for 41 hours. This removed most of the sugar and the acid and caused the suspended material to flock together. The clear juice obtained by filtering the dialysate was saturated with ammonium sulphate and left in the cold room over night. The flocky precipitate was filtered, washed carefully with saturated ammonium solution, and finally dissolved in about 150 cc. of water. A small insoluble residue was removed by filtration and the clear solution was dialyzed against running water with toluene for antiseptic until it was free from ammonium sulphate (6 days). The solution obtained by this procedure measured 350 cc. It was clear, faintly yellow, neutral to litmus, and gave no odor of cabbage until it was heated. It contained about 0.1 per cent of coagulable protein. Since this partially purified erepsin kept without apparent deterioration as long as was necessary for the purposes of this study, no attempt was made to obtain a dry preparation.

#### TRYPTOPHANE FROM THE DIGESTION OF WITTE'S PEPTONE.

That this "cabbage solution" contained an erepsin active over a considerable range of acidity and alkalinity is shown by the following experiment on Witte's peptone in which the criterion of digestion was the depth of the color obtained in the test for tryptophane.

Five cc. enzyme solution; 5 cc. 5 per cent Witte's peptone in 1 per cent sodium chloride solution; acid or alkali as indicated in the table; water to make a total volume of 12 cc; toluene; digested at 40°; tested after 17 hours and after 3 days.

To test for digestion, 2 cc. portions were removed, acidified with acetic acid when necessary, and treated with bromine water added drop by drop until a maximum color was obtained.<sup>1</sup> The table includes a digestion in which HCN was used as antiseptic.<sup>2</sup> 10 cc. portions of the enzyme solution after autolysis for 3 days, with toluene and with HCN respectively, did not give a trace of color when tested for tryptophane. The results are shown in the table on the following page.

The color test for tryptophane is not adapted to show small differences in the amount of digestion; but it is perfectly clear from the table that the erepsin of cabbage digests Witte's peptone over a considerable range of acidity and alkalinity. A concentration of

<sup>1</sup> It must be remembered that the color develops rather slowly.

<sup>2</sup> According to Vines, HCN favors the activity of cabbage erepsin (*Annals of Botany*, xvii, p. 249, 1903). For a discussion of the striking acceleration of papain-proteolysis by HCN, cf. Mendel and Blood: *This Journal* viii, p. 177, 1910.

ions corresponding approximately to that required to give action with methyl orange is distinctly inhibitory. Inas- he enzyme solution gave no tryptophane test after autoly- air to assume that all the tryptophane was formed from tion of the "peptone." Hydrocyanic acid apparently d certainly does not inhibit the hydrolysis.

*of Witte's Peptone by Cabbage Erepsin in Acid and Alkaline Solution*

ALKALI ADDED	TRYPTOPHANE TEST	
	After 17 Hours	After 3 Days
d; faintly al- to litmus.... aOH..... aOH; just al- to phenol- ein ..... aOH..... HCl; faintly litmus..... Cl..... HCl; just acid hyl orange... HCl..... ric acid; faint- to litmus.... itric acid..... itric acid..... ric acid; faint- d to methyl ..... er cent HCN; aintly acid to .....	Strong Strong  Strong Strong  Distinct Faint  None None  Distinct Faint None  None  Distinct	Deep Deep  Deep Deep  Deep Deep  Faint None  Strong Distinct Distinct  Very faint  Very deep

TYROSINE FROM PEPTONE "ROCHE."

he cabbage erepsin liberates tyrosine as well as trypto- m "peptone" is readily demonstrated by the use of pep- oche." This commercial product, recently commended

by Abderhalden and Schittenhelm<sup>1</sup> as a substratum for test for proteolytic activity, is so rich in peptids containing tyrosine that this amino-acid crystallizes out during the course of digestion.<sup>2</sup>

Five cc. of a 20 per cent solution of peptone "Roche," previously neutralized to phenolphthalein with sodium carbonate, were digested with 5 cc. of the cabbage erepsin solution in the presence of toluene at 40°. After 4 days a fine crop of crystals was obtained.

#### TRYPTOPHANE FROM CASEIN.

Like animal erepsin, cabbage erepsin hydrolyzes casein.

A suitable solution of sodium caseinate, only faintly alkaline to litmus, was prepared by dissolving 2 gms. of casein in 15 cc.  $\frac{N}{10}$  NaOH and making the solution up to a volume of 60 cc. with water. The slight excess of dissolved casein was removed by straining through cotton wool. 5 cc. of the solution digested with 5 cc. of the cabbage erepsin solution at 40° for 24 hours in the presence of toluene, gave a marked tryptophane reaction.

#### THE CLOTTING OF MILK

The solution of cabbage erepsin showed rennetic activity.

To test the milk clotting power of the enzyme solution a milk from which most of the fat had been removed by extracting with chloroform was used. To a series of tubes containing 2 cc. of this prepared milk were added 0.5 cc., 0.7 cc., 1.0 cc., and 2.0 cc. respectively of the enzyme solution. They were made up to a volume of 4 cc. each, put in a thermostat at 40° and examined at 15 minute intervals. The test with 2.0 cc. of casein showed incipient clotting in 30 minutes;<sup>3</sup> that with 1.0 cc. was clotting in 1 hour, and that with 0.7 cc. in 2 hours. The test containing 2 cc. of casein was kept in the thermostat for 48 hours, at the end of which time it gave a distinct tryptophane test, although the curd was by no means entirely solved.

<sup>1</sup> Abderhalden and Schittenhelm: *Zeitschr. f. physiol. Chem.*, 1 421, 1909.

<sup>2</sup> The tyrosine shows a considerable tendency to form supersaturated solutions.

<sup>3</sup> A comparable test on whole milk showed incipient clotting only after 4 hours.

## THE LIQUEFACTION OF GELATIN.

Cabbage erepsin liquefies gelatin.

To tubes containing 2 cc. each of "melted" 5 per cent gelatin (0.1 per cent thymol) were added 0.3 cc., 0.5 cc., 1.0 cc. and 2.0 cc. respectively of the erepsin solution, and water to make a total volume of 4 cc. When chilled in water after 4 hours digestion at 40°, only the test containing 2 cc. of enzyme solution remained liquid. The other tests still solidified in cold water after 20 hours digestion at 40°.

## THE DIGESTION OF FIBRIN AND COAGULATED EGG-WHITE.

The enzyme of the cabbage appears to be a typical erepsin. It does not digest fibrin or coagulated egg-white in acid, alkaline or neutral solution. Only the experiment with fibrin need be given in detail.

Five cc. cabbage erepsin solution; a small flock of fibrin; toluene; acid and alkali as follows: (1) none; (2) 0.5 cc. NaOH,—alkaline to litmus but not to phenolphthalein; (3) 1.0 cc.  $\frac{N}{10}$  NaOH,—alkaline to phenolphthalein; (4) 0.5 cc.  $\frac{N}{1}$  Na<sub>2</sub>CO<sub>3</sub>; (5) cc.  $\frac{N}{10}$  HCl, acid to litmus; (6) 1.0 cc.  $\frac{N}{10}$  HCl; (7) 0.5 cc.  $\frac{N}{1}$  HCl,—just acid to methyl orange; (8) 1.0 cc.  $\frac{N}{20}$  HCN. After a week's digestion at 40°, the fibrin was firm, and, except for swelling, apparently unchanged. The only exception to this was the fibrin in (7), which was soft and broke up when it was shaken. A control showed that this was attributable to the action of the acid alone. Tests for tryptophane were all negative.

## THE DIGESTION OF VEGETABLE PROTEINS.

It has already been stated (p. 219) that no tryptophane could be detected in the cabbage erepsin solution after it has been autolyzed for three days. Inasmuch as the solution gave the Adamkiewicz reaction, it is fair to infer that the erepsin was not able to hydrolyze the protein which was precipitated with it by ammonium sulphate.<sup>1</sup>

No evidence was obtained that the cabbage erepsin can hydrolyze hemp seed edestin in neutral, acid, or alkaline solution or in

<sup>1</sup> According to Vines (*Annals of Botany*, xvii, p. 249, 1903) a watery extract of cabbage leaves gives a distinct test for tryptophane after 4 hours autolysis at 40°.

the presence of HCN. The experiment, which was modelled ( that with fibrin need not be described in detail. A 3 per cent solution of edestin in 10 per cent sodium chloride solution w digested with an equal volume of the erepsin solution with neg tive results.

THE DIGESTION OF WITTE'S PEPTONE AT DIFFERENT TEMPER TURES.

Chittenden<sup>1</sup> and more recently, Delezenne, Mouton and Poz ski,<sup>2</sup> and Gerber<sup>3</sup> have shown that certain plant enzymes a not only comparatively resistant to high temperatures, but th they may under favorable conditions effect a very rapid hydroly at 70°, 80° or even 90°. Apparently the most favorable temper ture for the action of cabbage erepsin is not higher than that usua given for enzymes, 40°–50.°

*Experiment VII.* 5 cc. cabbage erepsin solution; 5 cc. 5 per cent Witte peptone solution; toluene; digested for 5 hours at the temperatures in cated in the table.

TEMPERATURE	TRYPTOPHANE TEST
20°.....	Faint
30°.....	Distinct
40°.....	Marked
50°.....	Marked
60°.....	Very faint
70°.....	Very, very faint

SUMMARY.

An active solution of a vegetable erepsin can be prepared fro the white cabbage by the ammonium sulphate method. Th solution deteriorates only slowly if kept in a cool place und toluene. Cabbage erepsin splits tryptophane from Witte's pe tone and casein, and tyrosine from peptone "Roche." It cl

<sup>1</sup> Chittenden, Joslin, and Meara: *Trans. Conn. Acad. of Arts and Sc* viii p. 281, 1892. Chittenden: *ibid.*, ix, p. 298, 1893.  
<sup>2</sup> Delezenne, Mouton and Pozerski: *Compt. rend. soc. de biol.*, lx, p 68 and 309, 1906.  
<sup>3</sup> Gerber: *ibid.*, lxvii, p. 318, 1909.

and liquefies gelatin. It does not digest fibrin, coagulated egg-white, or edestin in neutral, acid, or alkaline solution, or in the presence of HCN. It does not liberate any tryptophane from the protein precipitated with it from the cabbage by ammonium sulphate. The erepsin is active over a considerable range of acidity and alkalinity, but is inhibited by a concentration of hydrogen ions corresponding to acidity to methyl orange. No evidence was obtained that hydrolysis is favored by high temperatures or that it is greatly accelerated by HCN.<sup>1</sup>

For a discussion of the peculiarities of papain in regard to temperature and HCN cf. Mendel and Blood: *This Journal*, viii p. 177, 1910.





# A METHOD FOR DETERMINATION OF SACCHARINE<sup>1</sup> IN URINE.

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(Received for publication, May 10, 1910.)

In the summer of 1908, at Professor Folin's suggestion, I undertook to work out a method for the determination of the saccharine which appears in the urine after a dose of this drug has been taken. The following method is the outcome:

Proctor<sup>2</sup> in presenting a new method for the determination of saccharine, has discussed quite fully the previously known methods, so that, as no new ones have since appeared, it will not be necessary to make more than a passing reference to them here. They are briefly as follows:

1. Determination of the sulphur content by the alkaline fusion method.

2. Determination of the nitrogen, as ammonia, either by simple hydrolysis with dilute acids and distillation or by some modification of the Kjeldahl process.

3. Transformation of the saccharine into salicylic acid and its determination colorimetrically by the color given with ferric chloride.

4. Proctor's method<sup>3</sup> which depends on the fact that saccharine (and the related para-sulphamido-benzoic acid) liberates iodine quantitatively from a solution containing iodide and iodate. The iodine is then determined in the usual way.

Methods 1, 2 and 4 will give satisfactory results where a sufficient amount (0.5 gram or more) of saccharine is present in a fairly pure condition. With regard to method 3, Gannter<sup>4</sup> and

<sup>1</sup> This spelling is preferred because the term saccharin had been used previously by Peligot for another substance.

<sup>2</sup> *Journ. Chem. Soc. Trans.*, p. 242, 1905.

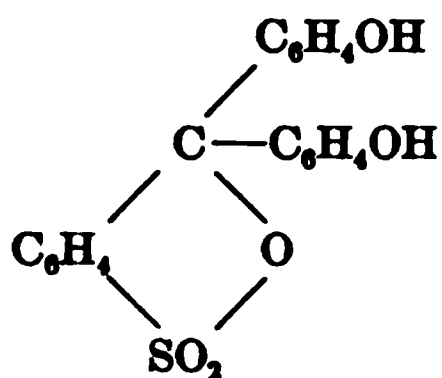
<sup>3</sup> Proctor: *loc. cit.*

<sup>4</sup> Gannter: *Zeitschr. f. anal. Chem.*, xxxii, p. 309.

Abraham<sup>1</sup> have shown that various organic substances, after fusion with sodium hydrate, will give colors with ferric salts, and there are difficulties connected with the quantitative transformation of saccharine into salicylic acid and the standardization of the color which render this method of little practical use.

An examination of the methods outlined above will show that none of them is suitable for this sort of work. Methods 1, 2 and 4 require more saccharine in a pure state than would be obtained from a whole day's urine. A method was required which would determine accurately the saccharine in a small amount of urine, say 10–25 cc.

The method here presented is a colorimetric one, depending on the transformation of the saccharine into what is probably phenol-sulphonephthalein or sulphurein, by treatment with a phenol-sulphuric acid mixture. The formula of this substance is



the nitrogen having been split off during the treatment with the strong mineral acid.<sup>2</sup> The color obtained is reddish in strong acid solution, purple-red in alkali and a bright clear yellow in weak acid solutions. Kastle<sup>3</sup> has made use of the purple color as a qualitative test for saccharine and other substances, but it fades somewhat on standing and was found difficult to match and so is not suitable for colorimetric work. The yellow color does not change appreciably in a month or longer so that it is possible to use it itself as a standard color solution.

It was first attempted to use the reaction directly on the urine, but the phenol-sulphuric acid reagent gives colors with so many

<sup>1</sup> Abraham: *Chem. Centralbl.*, i, p. 545, 1899.

<sup>2</sup> For a description and discussion of this and similar reactions see Remsen and co-workers: *Amer. Chem. Journ.*, vi, p. 180; ix, p. 372; xi p. 73; xvi, pp. 513, 528; xx, p. 263. Monnet and Koetschet: *Bull. de la soc. chim.*, iii, xvii, p. 690. Sisley: *ibid.*, p. 821.

<sup>3</sup> Kastle: *Chem. Centralbl.*, I, ii, p. 1574, 1906.

f the urinary constituents that the direct treatment had to be abandoned and an extraction made. In trying out the various solvents which have been suggested for the purpose it was found that benzol was the most satisfactory, in that it extracted the most foreign matter which interfered with the color. The benzol used must not leave any residue which gives a color with the reagent.

#### PROCEDURE.

*Extraction.* Urine containing about 3 milligrams of saccharine the amount may be easily calculated when it is remembered that between eighty and ninety per cent of the saccharine taken (excreted in the day's urine) is measured off into a 250 cc. Florence flask, evaporated to about 5 cc., cooled and strongly acidified with concentrated sulphuric acid (about 18 drops). Clean quartz sand is now added until the whole of the liquid is taken up. About 125 cc. of benzol is poured in and the whole boiled gently with a water-bath condenser (a glass tube about two feet long with the cork wrapped with tin foil) for two hours, with occasional shaking. The benzol is poured off and the extraction repeated with about 100 cc. of the solvent. After this extraction the solvent is poured off, the residue shaken out into a Buchner funnel, sucked dry and washed two or three times. The combined solvent with washings is set aside to cool to allow water to separate, then poured through a dry filter into a clean flask. The benzol is now distilled off, using a long glass tube as condenser, until only one or two cc. is left. This remnant must be removed carefully, with gentle heat and a slow current of air, because saccharine is readily volatile above 100°C.

*Conversion into the color.* To the flasks containing the saccharine extract is added about 5 cc. of the phenol-sulphuric acid reagent and the whole heated at a temperature of 140°–150° for two hours. This temperature must be quite accurately maintained, since the speed of reaction drops off very rapidly below 140°, while at much above 150°, although the reaction goes more rapidly, there is danger of overheating, with destruction of part of the color.<sup>1</sup> The flask is then cooled somewhat and about 150 cc. of hot water is added

<sup>1</sup> This is slightly higher than the temperature limits found by Sohon: *Amer. Chem. Journ.*, xx, p. 262, but the period of heating is much shorter.

After the syrupy residue is dissolved the solution is neutralized by the addition of sodium acetate crystals in slight excess, indicated by the disappearance of the pinkish tinge, leaving the liquid yellow. The mixture is allowed to stand some hours or over night.

*Reading.* The neutralized liquid is transferred to a graduated flask and diluted up to the mark. A small portion (25–50 cc.) is filtered off for colorimetric reading. The filtrate should be clear yellow, with not more than a trace of turbidity. It is then compared with the standard color in a Duboscolorimeter, setting the standard at 20 mm.

### *Solutions Required.*

1. **THE REAGENT.** Equal parts by weight of concentrated sulphuric acid and pure crystallized phenol (equimolecular portions with 5 per cent excess of phenol) are stirred until dissolved. The solution should be only slightly colored. For a blank determination with the reagent should give only a faint tinge to the solution. If crystals separate on standing they should be re-dissolved by gentle warming.

2. **STANDARD COLOR SOLUTION.** Weigh out accurately 3 mg. of pure saccharine into a 250 cc. flask, add 5 cc. of the reagent and digest at 145°–150° for two hours. Dissolve in water and neutralize with sodium acetate and make up to 500 cc. Check the accuracy of the solution by comparison with other similar amounts of saccharine. These should check to within 1 per cent.

### EXPERIMENTAL RESULTS.

#### I. *Testing with pure dry saccharine.*

Weight of Saccharine Taken.	Weight of Saccharine Found.
0.0028	0.0029
0.0054	0.0053
0.0031	0.0032
0.0054	0.0053
0.0034	0.0036
0.0058	0.0058
0.0051	0.0050
0.0038	0.0039
0.0017	0.0016

*Extraction from Urine.*

- a. Urine containing 0.1 gm. saccharine per liter; 25 cc. taken for determination, containing 0.0025 gm. saccharine.

Found: 0.0025 0.0023 0.0024 0.0025 gm.

Ave. 0.0024 gm.

- b. Urine containing 0.4792 gm. saccharine per liter; 5 cc. taken = 0.0023<sup>9</sup> gm. saccharine.

Found: 0.0024<sup>3</sup> 0.0023 0.0024 0.0022<sup>7</sup> gm.

Ave. 0.0023<sup>4</sup> gm.

- c. Urine containing 0.2 gm. per liter; 20 cc. taken = 0.0040 gm. saccharine.

Found: 0.0039 0.0039 0.0040 0.0039 gm.

Ave. 0.0039 gm.

The results of these experiments show that small amounts of charine may be determined in the urine by this method with a sonable degree of accuracy.

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# ESTIMATION OF SACCHARINE IN URINE AND FECES.

By ALFRED J. WAKEMAN.

*(From the Laboratory of Dr. C. A. Herter, New York City.)*

(Received for publication, July 1, 1910.)

The main features of the method for the determination of saccharine in urine as reported by Bloor in the preceding article, were kindly given to me by Dr. Folin as I was about to start a series of experiments involving the quantitative estimation of saccharine in human excreta. In the course of the work I took occasion to modify the procedure in more or less essential details, and the method finally adopted is given below.

The main points of difference from the procedure reported by Bloor are the use of ethyl acetate as a solvent instead of benzol and of lead acetate in the place of sodium acetate, and the use to some extent of different apparatus. The method in detail as applied to urine is as follows:

An amount of clear urine containing from 3 to 4 milligrams of saccharine is accurately measured into a 150 cc. pear-shaped, short-necked separatory funnel. Sodium chloride is added to nearly the point of saturation or about 3 grams to 10 cc. of fluid. One cc. of 50 per cent sulphuric acid is added and the mixture is shaken out with 25 cc. of ethyl acetate.<sup>1</sup> The ethyl acetate solution is washed with 3 cc. of saturated sodium chloride solution. The latter is allowed to drain into the urinary mixture, and the washed ethyl acetate solution is poured into a side-neck test tube twelve inches long and having an inside diameter of one and one-quarter inches. The urinary mixture is shaken out the second time with 15 cc. of ethyl acetate and the ethyl acetate solution washed as before with 3 cc. of saturated sodium chloride solution, the latter

<sup>1</sup> The solvent employed was the ordinary ethyl acetate and contained 7 per cent of alcohol.

being allowed to drain into the urinary mixture and the washed ethyl acetate solution being poured into the test tube with the first extraction. This operation is repeated the third time.<sup>1</sup> The test tube containing about 55 cc. of the washed ethyl acetate extraction is attached to a condenser and immersed in a water bath kept at about 90°C. by a thermo-regulator. After the ethyl acetate is distilled off to about 4 cc. the side neck of the test tube is connected with suction, a rubber stopper containing a drawn-out tube having been placed in the test tube. A stream of air is thus allowed to play toward the bottom of the tube which is still immersed in the water bath. This insures a complete and rapid removal of the last traces of moisture without danger of overheating. Five cc. of the warmed phenol sulphuric acid reagent, prepared by bringing together equal weights of crystallized phenol and sulphuric acid, are now added to the test tube, preferably by means of a 5 cc. pipette with a long rubber tube attached.

The inner walls of the test tube are moistened with the reagent and the test tube is then immersed about two inches into a paraffin bath kept at 150°C by the thermo-regulator. A rack composed of two pieces of board about three inches apart with one and one-half inch holes bored through the upper and lower board and placed on top of the bath forms a convenient receptacle for the side-neck tubes. The tube covered with a porcelain crucible cover is kept in the bath two and one-quarter hours. The contents while still warm are then somewhat diluted with hot water and transferred after cooling to a 500 cc. measuring flask. Thirty cc. of a 40 per cent solution of lead acetate are added and the flask filled to the mark and shaken.

Lead acetate not only renders the fluid less strongly acid but the precipitated lead sulphate carries down with it the ordinary urinary coloring matter, leaving a filtrate practically colorless. The yellow dye derived from the saccharine is not affected.

A portion of the contents of the flask is filtered through a double thickness of No. 597 Schleicher and Schüll 12.5 cm. filters and the perfectly clear yellow fluid is matched in a Duboscq colorimeter

<sup>1</sup> 0.2233 gram of saccharine in 25 cc. water yielded 0.2024 gram of saccharine or 90 per cent upon the first extraction, 0.0160 gram, or 7 per cent upon the second extraction and 0.0067 gram or 3 per cent upon the third extraction.



Standard solution prepared at the same time and in a similar manner from a small volume of normal urine to which 3 milligrams of pure saccharine had been added.

#### METHOD APPLIED TO THE FECES.

The saccharine content of feces may be determined by taking a definite amount of the finely divided dried feces—say 2 grams—and digesting at a gentle heat with about 200 cc. of 50 per cent alcohol saturated with sodium bicarbonate. The fluid is allowed to settle to about 50 cc., transferred to a 200 cc. measuring flask, topped to the mark with water, shaken and filtered. To the filtrate 5 cc. of dilute sulphuric acid and 30 grams of potassium iodide are added and the solution diluted with water in a 250 cc. flask to 125 cc. The contents are shaken and filtered. Ten cc. of the clear filtrate, representing one-fifth of the feces taken are used and shaken out with ethyl acetate. The procedure from this point is the same as with the urine.

Standard solution may be prepared by adding a known amount of pure saccharine to a definite amount of dried normal feces and proceeding in the same manner as with the unknown sample.

#### RESULTS.

0.003 gram saccharine was dissolved in 1000 cc. of urine. Thirty cc. of this standard solution, containing 0.0009 gram saccharine were taken for extraction.

Recovered,	I	0.00309
	II	0.00301
	III	0.00294
Average,		0.00301

0.004 gram saccharine was dissolved in 1000 cc. of urine. Twenty cc. of this standard solution, containing 0.0008 gram saccharine were taken for extraction.

Recovered,	I	0.00395
	II	0.00394
	III	0.00370
Average		0.00386

0.004 gram saccharine was dissolved in 1000 cc. of urine. Ten cc. of this standard solution, containing 0.0004 gram saccharine, were taken for extraction.

Recovered	I	0.00407
	II	0.00400
	III	0.00397
Average		0.00401

*Feces.*

0.25 gram saccharine was added to 109.3 grams of fresh normal feces. The weight of the feces dried was 15.1 grams. The saccharine content 100 grams of the dried feces would therefore be 1.655 grams.

Upon extraction,

I. 2.0069 grams of the dried feces yielded 0.03312 gram saccharine equivalent to 1.650 grams in 100 grams of the dried feces.

II. 1.9835 grams of the dried feces yielded 0.03128 gram saccharine equivalent to 1.577 grams in 100 grams of the dried feces.

The standard for comparison was prepared from 20 cc. of urine containing 0.003 gram saccharine.

## MANGANESE OF THE TISSUES OF LOWER ANIMALS.

By H. C. BRADLEY.

(From the Department of Physiology of the University of Wisconsin,  
Madison, Wis.)

(Received for publication, June 23, 1910.)

In 1907 a preliminary note was published recording the finding of manganese in the tissues of fresh water mussels obtained from the Madison Lakes.<sup>1</sup> It was found that specimens of the thin shelled *Anadonta*—the typical lake form—contained approximately 1 per cent of manganese in the dry tissues. The eggs and embryos contained the element to the extent of 0.6 to 0.8 per cent. It was believed that this would eventually be found a normal characteristic not only of mussels from waters adjacent to Madison, but of the entire species in its very extended distribution. Sufficient data are now available to confirm this point, at least so far as the North American varieties are concerned. It is obvious that a single normal individual lacking manganese would cast grave doubts upon the view that the element is itself a normal and necessary one to this group of animals. We have, therefore, examined many hundreds of individuals during the last three years, and we have not yet found a specimen in whose tissues manganese was not abundant. Furthermore the quantitative results presented below show that manganese is present in rather uniform amount, showing no large fluctuations as would be expected were the element an adventitious one.

The specimens represent a wide range of habitat, covering practically that portion of the United States east of the Rocky Mountains, and the St. Lawrence basin in Canada. Knowing the ease with which the *Unionidæ* can migrate parasitic in the gills of fishes, there can be no reason for doubting that the mussels

<sup>1</sup> Bradley: this *Journal*, iii, p. 151, 1907.

of the Hudson Bay basin and of the Pacific Coast are essentially the same as those we have examined to the south and east. The watershed north of the St. Lawrence drainage is a low one with many points where water connection between the two basins is complete, and where small fish bearing the parasitic embryos of the mussels, may pass from one side to the other. The same is true also of the divide between the Mississippi basin and the Pacific drainage. We feel safe therefore in including the whole of North America at least, in a generalization based upon the specimens we have examined. Not only is manganese an element which is normal and necessary in this family of molluscs as it is to-day but we have good evidence to conclude that it has been a normal element in past geologic eras, and that it is therefore a characteristic of the most fundamental kind, surviving the changes of habitat and environment incident to its existence over an extended period of time.

In collecting the data presented here two general methods of analysis were adopted. The modified Volhard process as outlined in the preliminary note was used for all of the earlier analyses and has frequently been used as a check on the second method. The Volhard process requires a preliminary ashing of considerable amounts of material—a process which is at best somewhat uncertain and tedious. The method is useless in determining very small amounts of the element. The second method while not quite so accurate perhaps as the first where large quantities of material are available, is much more rapid and has the advantage of requiring very little material. The method is based upon the reaction in nitric acid solution of ammonium persulphate and salts of manganese to form permanganic acid, and the subsequent titration of the latter. With such modifications as are required to make the reaction applicable to tissues we have followed the method advocated by H. Procter Smith<sup>1</sup> for estimating manganese in steels. The details of the method as we have used it are as follows:

About 0.200 gram of the dry tissue powder is weighed out in a platinum crucible, allowing an error of 0.0005 gram. The crucible is gently heated till the organic matter has largely burned off. Potassium nitrate is then

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<sup>1</sup> H. Procter Smith: *Chem. News*, xc, p. 237.

duced a little at a time, avoiding a violent reaction, until the carbon is completely oxidized and a homogeneous melt remains. The crucible is brought to a dull red heat giving a clear green melt if manganese is present. When cool, the melt is dissolved in boiling dilute nitric acid, the crucible removed and rinsed carefully into the beaker containing the rest of the solution. Ten cc. of concentrated nitric acid are added and the total volume of about 75 cc. approximated. The solution is brought to the boiling point and 2 grams of ammonium persulphate added cautiously. As the solution stands almost boiling a 10 per cent silver nitrate solution is added drop by drop to catalyze the reaction, until a sudden clearing of the mixture indicates that sufficient silver salt is present. Ammonium persulphate is added and the solution boiled till the reaction has nearly reached its completion. The permanganic acid solution is then cooled to the temperature of tap water and titrated with a standard solution of arsenious acid. From the known strength of the latter the amount of manganese in the tissue can readily be calculated. The entire analysis can be made in fifteen minutes, while the preparation of the ash sample for the Volhard method requires half to three-quarters of an hour at least.

It is quite obvious that in a comparative investigation of this method absolute accuracy is not of import. The variations of individual specimens from the same locality, the errors of sampling, etc., are of greater magnitude presumably than the errors of the analytical process. It is not significant therefore if the persulphate method finds 1.20 per cent of manganese in a tissue known to contain 1.30 per cent, for such a difference could have no meaning biologically. With the persulphate method it becomes entirely practicable to dissect out the tissues of a single mussel and make analyses on these tissue fractions. It will readily indicate manganese down to 0.00005 gram, and it will determine with a fair degree of accuracy the element between the limits of 0.0001 and 4 gram. Above this amount the method is impracticable because it is found difficult to get a perfect melt with all of the metal in solution.

In table I will be found some of the results of a series of determinations made upon material from the Madison region collected during the years 1906 to 1909. The data previously published are included in the table for the sake of completeness.

Attention should be called to the following points in the table: The fluctuations in manganese of ash and tissue are large—6.0 per cent of ash. The variations of manganese in tissue are

TABLE I.

NO.	DATE.	SPECIES.	ASH.	Mn IN ASH.	Mn IN TISSUE.	N TISS.
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	October '06	Mixed	21.63	4.20	0.931	
2	October	Mixed	26.00	4.60	1.19	
3	October	Mixed	21.94	4.2	0.929	
4	October	Mixed	17.88	5.7	1.02	
4(a)	October	eggs	39.55	1.5	0.633	
5	October	Mixed	13.88	4.3	0.601	
5(a)	October	eggs	35.20	2.3	0.818	
6	April '07	Anadonta	25.0	4.9	1.225	
7	April	Unio	21.5	5.0	1.075	
8	April	Unio	19.5	5.0	0.975	5.3
9	April	Anadonta	22.0	4.9	1.078	
10	May	Mixed	19.0	4.5	0.855	
11	May	Anadonta	18.0	5.2	0.936	
12	May	Unio	21.0			5.6
13	May	An. 4-6''	25.0	4.8	1.20	6.4
14	May	An. 3-4''	18.5	4.5	0.832	6.1
15	May	An. 1-3''	17.5.	4.1	0.718	6.1
16	May	Unio	24.5	4.5	1.103	
17	June	Unio	21.0	6.0	1.26	
18	June	Anadonta	23.5	4.8	1.128	
19	June	Mixed	15.0	5.5	0.825	7.
19(a)	June	Tis. fluids	10.0	3.2	0.32	
20	June	Anadonta	21.5	5.5	1.183	
21	June	Unio	20.0	5.2	1.040	
22	August	Anadonta	11.5	4.2	0.483	
23	August	Unio	21.0	4.4	0.924	
24	October '09	Mixed			1.510	
Average.....			21.8	4.52	0.95	6.

closely parallel to the variations in the ash, and in a general both increase with an increase of ash in the tissue. In the same cality, and under apparently identical conditions increasing age size leads to a corresponding increase in ash, per cent of mangar in the ash and in the tissue.<sup>1</sup> There is no appreciable differe between the two groups when taken from the same locality. Th

<sup>1</sup> Nos. 13, 14, and 15 were sorted into three sizes as indicated in the ta they thus represent three ages.

taking an average of the normal specimens of *Anadonta* and *Unio* separately, one obtains the following figures:

	ASH.	Mn. OF ASH.	Mn. OF TISSUE.
		<i>per cent</i>	<i>per cent</i>
<i>Anadonta</i> .....	21.3	4.8	1.04
<i>Unio</i> .....	21.2	5.02	1.06

There seems to be no evidence of a definite seasonal fluctuation of any of the components of the tissue.

Table II contains the results obtained from samples of mussels from the Mississippi River near Galena, Illinois; nearly all were the very heavy shelled varieties dredged for the button factories. Each sample consisted of a dozen or more specimens of a single variety.

TABLE II.

NO.	DATE.	SPECIES.	ASH.	Mn IN ASH.	Mn IN TISSUE.	N IN TISSUES.
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	August '07	<i>Unio</i>	16.0	4.3	0.688	7.11
2	August	<i>Unio</i>	16.0	3.6	0.576	
3	August	<i>Anadonta</i>	18.5	4.2	0.777	7.37
4	August	<i>Unio</i>	23.5	3.5	0.823	
5	August	Mixed	13.5	3.9	0.526	
6	August	<i>Unio</i>	16.5	3.7	0.611	
7	August	<i>Anadonta</i>	13.5	3.7	0.500	
8	August	<i>Unio</i>	13.5	4.2	0.567	
9	August	<i>Unio</i>	17.5	4.2	0.735	
	Average.....		16.5	3.9	0.63	7.24

Table III contains the data from mussels obtained in the Wisconsin River, and from the Temagami Forest Reserve of Ontario Canada. Specimens from these regions show the smallest inorganic and manganese content of any that we have found.

TABLE III.

NO.	DATE.	SPECIES.	ASH.	Mn IN ASH.	Mn IN TISSUES.	P
<i>Wisconsin River.</i>			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	September '07	Mixed	14.5	2.4	0.348	8
<i>Temagami Reserve.</i>						
1	August '08	Anadonta	18.5	3.5	0.648	9
2	August	Anadonta	18.5	3.8	0.703	
3	August	Unio, liver	14.0	3.6	0.504	
3	August	Unio, fibrous	6.0	5.3	0.318	
4	August	Unio	13.0	2.3	0.299	
5	August	Anadonta	14.5	2.8	0.406	9
6	August	Anadonta	13.5	2.2	0.292	9
7	August	Anadonta	16.0	3.3	0.528	10
Average.....			15.4	3.1	0.45	9

. Table IV was obtained from mussels collected from various parts of the Mississippi basin through the courtesy of the Bureau of Fisheries. They represent lakes and streams of southern Wisconsin, Indiana, Iowa, Michigan, and Illinois. In preparing the samples for analysis it was noticed that one could often separate the mixed tissues by sifting, into a light fibrous fraction derived from muscles and connective tissue, and a more solid, easily pulverized fraction derived from the liver, reproductive glands. The fibrous fraction constitutes but a small part of the total weight of the mussels. We have therefore divided many of our mixed samples by sieves and the figures in the table indicate usually that the more easily pulverized tissues—that is, the fraction passing the 100-mesh sieve—is always the fraction richest in inorganic salts and manganese. We have not however attempted to determine what per cent of the total body weight is represented by each fraction, so that the average of the figures is of less significance than it should be.

In Table V are given the results obtained on a lot of mussels from near Madison. Forty or fifty average specimens were selected from several hundred collected, and after killing in boiling water the tissues were separated into the following fractions: muscle (both adductors and pedal), eggs (from several of the ripe females)



TABLE IV.

NO.	DATE.	SPECIES AND FRACTION.	ASH.	Mn IN		N IN	
			per cent.	ASH.	per cent.	TISSUE.	per cent.
1	June '09	Unio	.....	.....	1.2	.....	.....
2	June	Unio	23.5	3.81	0.896		
3	June	Unio	15.0	2.67	0.400		
4	June	Unio	21.0	4.84	0.948		
5	July	Anadonta	18.5	3.5	0.664		
6	July	Unio	15.5	4.7	0.738	7.65	
7	July	Unio	12.0	3.8	0.464	7.28	
8	July	Anadonta	15.5	5.8	0.875		
9	July	Anadonta	12.5	8.2	1.025		
10	July	Anadonta	17.0	3.4	0.590		
11	July	Anadonta	17.5	3.5	0.620		
12	July	Anadonta	12.5	4.3	0.538		
13	July	Anadonta	13.5	4.1	0.547		
14	July	Anadonta	12.0	3.5	0.422		
15	July	Anadonta	13.0	6.6	0.865		
16	July	Anadonta	24.5	6.1	1.51		
17	July	Unio: 10 mesh	11.0	3.83	0.421		
18	July	80 mesh	13.0	4.05	0.527		
19	July	100 mesh	13.0	4.30	0.558		
20	July	Unio: Mixed tissue	14.5	6.4	0.938	7.11	
21	July	Unio: Muscle fibre	11.0	6.3	0.695		
22	July	20 mesh	8.5	4.5	0.390	11.88	
23	July	40 mesh	8.5	3.7	0.316	11.06	
24	July	60 mesh	10.0	4.85	0.485	10.41	
25	July	80 mesh	12.0	4.65	0.558	10.8	
26	July	100 mesh	23.5	5.73	1.349	8.33	
27	July	Unio: mixed tissue	15.0	5.12	0.769		
28	July	fibrous tissue	7.0	5.87	0.411		
29	July	60-80 mesh	16.0	5.8	0.927		
30	July	100 mesh	21.0	4.76	1.001		
31	July	Unio: mixed tissue	9.5	5.32	0.506		
32	July	100 mesh	13.0	5.6	0.738		
33	July	Unio: mixed tissue	15.0	3.8	0.569	7.35	
34	July	fibrous tissue	5.5	5.5	0.306	11.28	
35	July	100 mesh	23.5	5.2	1.222	5.24	
36	July	Anadonta: 20 mesh	9.5	4.1	0.390		
37	July	40 mesh	12.0	4.8	0.580		
38	July	60 mesh	16.0	4.4	0.708		
39	July	80 mesh	18.5	5.0	0.930		
40	July	100 mesh	28.0	3.9	1.034		
41	July	Unio: muscle tissue	2.0	1.69	0.034		
42	July	liver and ovary	10.0	2.52	0.252		
43	July	mantle and gills	27.5	4.36	1.199		
44	July	mixed tissues	.....	.....	0.423	.....	

nephridial organs, mantle, gills, liver (including the reproductive organs associated with the hepato pancreas) and stomach. The latter is undoubtedly contaminated with food residues and adhering portions of the liver mass. When dry it separated into a small fibrous portion and a fraction easily reduced to a fine powder.

TABLE V.

TISSUE.	ASH.	Mn IN ASH.	Mn IN TISSUE.	N IN TISSUE.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Muscle.....	6.0	4.87	0.293	11.48
Eggs.....	37.0	2.024	0.749	10.37
Stomach; fibrous part.....	14.5	5.73	0.831	
Stomach; non-fibrous.....	32.0	4.66	1.492	6.1
Nephridial organs.....	27.0	5.31	1.434	7.58
Gills.....	33.5	4.89	1.638	6.32
Mantle.....	48.0	5.12	2.457	5.45
Liver.....	39.0	5.85	2.107	5.56

Noteworthy is the exceedingly high mineral content of the mantle. Its ash exceeds that of ordinary mammalian bone though it is a perfectly soft and pliable sheet of tissue. It is a type of structure quite different from anything we find among vertebrate tissues. It is reasonable to suppose that the enormous mineral content of the mantle bears some relation to its activity in shell construction, and this is born out further by the observation that the mantle of *Unio* is a thicker, more substantial organ than that of *Anadonta* and is richer in ash. *Unio* secretes a much heavier shell than *Anadonta*. The function of the high mineral content of the gills is not apparent—they too are in a class with osseous tissue in this respect. It is to be noted also that the nitrogen of tissues bears an inverse relation to the ash; in no tissue does it reach the level of nitrogen in a typical protein.

It seems probable that the function of the manganese is a respiratory one. Its abundance and presence in every tissue and organ of the body is suggestive of some such fundamental function in the metabolism of the mussels. The fact that no one individual among the several hundred examined lacked the element or even

owed its presence in small amounts speaks strongly against an adventitious occurrence. The presence of manganese in the eggs is another argument against its adventitious nature. One point seems to present difficulty to the acceptance of the hypothesis that manganese serves in a respiratory rôle, and that is the small amount present in muscle tissue. It is a matter of common observation that active muscles are frequently provided with an extra respiratory mechanism. Thus we find in vertebrate heart muscle and in those skeletal muscles most actively employed a higher content of hæmoglobin and of iron than in less active muscles. Among the molluscs we find cases where an animal provided with a copper-containing circulatory fluid has the muscles of its heart and radula pigmented with hæmoglobin as though a special provision for the respiration of those more active contractile organs. The muscles of the *Unionidæ* however contain less manganese than any other tissues. It must be born in mind however that the muscles of these clams are extremely inactive. The pedal muscle is hardly more than rudimentary, and when used in propelling the animal it makes a single contraction followed by a long period of many minutes of relaxation. In the same way the adductors are for most part relaxed, allowing the valves to lie slightly open, with an occasional contraction pumping out the water. The muscle tissue therefore of these animals has an extremely low order of activity—quite unlike the continued rhythmic contraction of the heart or radula of *Sycotypus* for example. The slight provision for respiration of these tissues is therefore not so difficult to understand.

If we grant the normality of manganese in these mussels and its respiratory function, the question arises as to its source. Practically all soils and rocks contain traces of the metal, and it is thus present in most waters. In regions like Wisconsin where limestones are abundant and rather rich in manganese the waters are comparatively rich in the element.

But even in the most favorable localities the actual amount of the element is exceedingly small. One does not however have to presuppose an enormous concentration of an element of this kind by the mussels themselves, for the natural food of the *Unionidæ* is highly manganiferous. Wherever we have found the mussels living we have also found the brown masses of

*Crenothrix* with diatoms and infusoria that are probably rich manganese. *Crenothrix* is well known as an organism which concentrates manganese from the water in which it lives to such extent that its filaments are frequently covered thick with brown layer of hydrated oxide of manganese. A marine diatom has recently been described which also seems to effect a similar concentration of the element,<sup>1</sup> and it seems highly probable that there are many other low forms of life associated with *Crenothrix* in fresh waters which have the same function. In any case it is easy to demonstrate that manganiferous organisms are associated with the mussels, by keeping the latter in aquaria. The tanks soon slimy with the brown masses of the manganiferous *Crenothrix*. These may be grown in quantity for analysis, by keeping fresh running water in the tanks and from time to time collecting the slime. We have thus removed crop after crop of such material aggregating many hundred grams, from water which contained not more than 0.0000066 per cent of the metal. The following table shows the analyses of samples of this material obtained at different times and under different conditions. The material was boiled with alcohol and ether and dried at 105°:

TABLE VI.

NO.	MATERIAL.	ASH IN TISSUE.	Mn IN TISSUE.	N IN TISSUE
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	Bottom-slime	52.0	0.337	3.20
2	Bottom-slime	52.0	1.065	
3	Bottom-slime	76.0	1.351	
4	Bottom-slime	58.0	0.275	1.33
5	Bottom-slime	56.0	0.128	2.73
6	Surface-growth	40.0	1.840	3.66
7	Surface-growth	24.0	0.907	1.54

When the mussels are kept in shallow glass vessels with a change of water once a day, they are placed practically under starvation conditions. The stringy masses of excreted material may thus be obtained free from contaminating food. It is noteworthy that under such conditions manganese is abundantly excreted at first.

<sup>1</sup> Jaroslav Peklo: *Österreichische Botanische Zeitschr.*, lix, p. 289.

the material is brown in color like the food, and contains about the same amounts of inorganic salts and manganese. One such sample excreted after one day of starvation showed 51.0 per cent ash, and 0.337 per cent of manganese. Gradually the manganese decreases, however, the excreta become white or light yellow, and after a week or ten days no more manganese can be detected. This would seem to indicate that under normal conditions the mussels are in a manganese balance—as much is given off in the excreta as is obtained from the food. If, however, manganese starvation is maintained a very definite retention of the element by the organism results. Furthermore in early spring after several months of starvation the tissues of the mussels are not appreciably poorer in manganese than they are in midsummer when *Crenothrix* is most abundant.

It is of interest to note that in many of the Canadian lakes the mussels could not be found. In such lakes the water was clear, the bottom was apparently free from the brown masses of *Crenothrix*, and the exposed stones showed none of the black or brown stain of iron or manganese so common in most localities. Wherever the mussels were encountered, all of these evidences of manganese were also found. We feel convinced that the mussels will not be found in waters which will not support *Crenothrix* and the associated infusoria and diatoms. The mussels of the Temagami region were invariably small, and they are poor in manganese; the mussels of the Mississippi basin are often of great size, their tissues are richest in manganese, and they are very abundant. There seems to be a correlation between the richness of the manganeseiferous food of the water and the size, abundance and content of manganese of the clams.

Table VII gives some miscellaneous results, of which the first two are of chief importance:

TABLE VII.

MATERIAL.	MANGANESE.
	<i>per cent</i>
Shell, <i>Unio</i> ; fresh.....	0.148
Shell, <i>Unio</i> ; fossil.....	0.085
Boiler scale, Madison.....	0.20
Water; Lake Mendota.....	0.0000066

A number of qualitative tests have shown that the *Union* secrete a shell the nacre of which contains manganese. It seems to be as characteristic as the presence of the element in soft parts. It furnishes therefore a means for the study of *Unionidæ* of previous geological epochs. Through the court of Dr. George Wagner we have secured some perfectly preserved nacre, white and lustrous as a fresh shell, from a specimen antedating the Pliocene period of Wisconsin.<sup>1</sup> Analysis showed manganese in nearly normal amount. We can conclude therefore that *Unionidæ* have utilized manganese physiologically for a greatly extended period of time. If the species developed from a marine form it is quite probable that this marine ancestor was also a manganeseiferous animal.

This brings up the question as to whether marine molluscs of the present time utilize this element. A single case is on record in the literature—*Pinna squamosa*, investigated by Griffiths in 1892,<sup>2</sup> and found to have a blood protein rich in manganese. Since that time so far as we are aware, no one has either confirmed the observation or looked further for a physiological occurrence of this unusual element. It is highly improbable that one or two species of molluscs should have so marked a metabolic idiosyncrasy with no indication of it in allied forms. A short reconnaissance of the common bivalves of the Woods Hole region of Massachusetts was therefore carried on during the summer of 1909. Of the specimens then readily obtainable at least one seemed to regularly contain manganese, while others frequently showed its presence.

In *Modiola modiolus* we always found manganese, even after three weeks of starvation; never in such abundance as it occurs in the tissues of the *Unionidæ*. All of the tissues reacted positively for the metal, but the brown nephridial organs were by far the richest. In *Pecten* the results were not uniform. In some specimens the element was abundant, while in others it occurred only in traces. The stomach contents were frequently rich in manganese, so that probably the presence of the element in

<sup>1</sup> The description of this fossil shell can be found in *Nautilus*, x, p. 97. The specimen is apparently identical with *Unio crassidens* of the southern waters of the present time.

<sup>2</sup> Griffiths: *Comp. rend. de l'acad. des sci.*, cxiv, p. 840, 1892.

tissues was an adventitious one depending upon locality and diet at the time of collection. In *Mytilus edulis* manganese was present in all specimens examined, but in mere traces so that no significance can be attached to it. In other bivalves it was occasionally found in traces. It is our intention to continue this line of investigation as opportunity permits and extend, if possible, our knowledge of the utilization of manganese in the lower animals.





## SOME LIPASE REACTIONS.

By H. C. BRADLEY.

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Nearly every investigator of enzyme reactions has found that some sort of relation exists between the amount of enzyme employed and the degree to which hydrolysis proceeds. This proportionality constitutes an apparent difference between enzymes and true catalytic agents. The latter accelerate reactions but do not modify equilibria. Enzymes do apparently modify the equilibria of reactions which they accelerate. Whether or not a real difference shall prove to exist between enzymes and true soluble catalytic agents, it is believed that data upon this point may be worth presenting. The problem arose incidentally during a study of human pancreatic juice, but its direct bearing upon the theories of digestion, absorption and tissue synthesis led to a more detailed examination of this particular point.

It is obvious that the more simple the hydrolytic reaction induced by an enzyme the easier it becomes to draw conclusions from the facts of the reaction. For this reason lipase is an enzyme which is ideal for such an investigation as this. The hydrolysis of an ester in the presence of an excess of water is a monomolecular reaction provided the products are removed. The products themselves undergo no molecular rearrangements, so that reversion to the original ester is theoretically and actually possible. The analytical processes involved are rapid and exact so that a close scrutiny of the reaction is possible throughout its course. The pancreatic juice which was used as a source of lipase has been fully described elsewhere.<sup>1</sup> Suffice it to say here, it was obtained by temporary fistula, was very active lipolytically, alkaline to litmus with sodium

<sup>1</sup>H. C. Bradley: *this Journal*, vi, p. 133.

bicarbonate, as a rule clear, and under toluol it retained its lipolytic power unimpaired for several months. It made an excellent basis therefore, for a study of lipase reactions. Both ethyl butyrate and triolein were used in the digestions. The former has the apparent advantage of forming a true solution, the latter of being a common physiological ester. No real difference has been observed in the digestions of the two esters and we shall report here only the experiments upon triolein. Emulsions of an oil, as they become more perfect must approximate more and more closely to a solution as the size of the masses of oil approach molecular dimensions. That the average total surface presented is the same under the same conditions is abundantly proven by the concordance of duplicate digestions. The regularity of the curves and their similarity to those obtained with a soluble ester indicate that objections to a reaction involving a suspension, rather than a solution of the zymolite, are of no significance in this reaction. The system is presumably already heterogeneous since the lipase is in colloidal solution.

Two facts have led to the belief that enzymes are true catalytic agents. First, because a minute amount of the enzyme produces such large results in the way of hydrolysis. Second, because it is not used up in the reaction. The first point is not open to discussion; the second may perhaps be considered still unsettled though the consensus of opinion would undoubtedly agree with the statement above. Enzymes undoubtedly disappear in the course of digestions, but this is thought to be due to a secondary destructive action of one of the products upon the enzyme. Some investigators still believe a permanent combination between enzyme and zymolite occurs. While we shall prove that in the case of lipase no such permanent combination takes place with triolein, it is nevertheless equally certain that the regular increase of the enzyme present in digestion results in as regular an effect upon the final equilibrium attained in the reaction. Some authors have found what they believe to be a linear relation, others have found the relation best expressed by a simple logarithmic function, as in the so-called "law of Schütze." We shall show later that neither expression is correct though both may approximate the truth in certain very limited stages of the reaction. A typical example of the relation between enzyme, zymolite, and digestion is given in the following experiments.

EXPERIMENT I. Glass stoppered bottles of about 200 cc. capacity filled with the following digestion mixtures:

	TRIOLEIN.	WATER.	JUICE.	TOTAL VOL.
	cc.	cc.	cc.	cc.
.....	2	13	125	140
.....	4	11	125	140
.....	6	9	125	140
.....	8	7	125	140
.....	10	5	125	140
.....	15	0	125	140

The sample used was a 24-hour sample diluted with ten volumes of Triolein was added last to each bottle, and after shaking thoroughly an aliquot was withdrawn and titrated in alcoholic solution with alcoholic  $\frac{N}{10}$  KOH, using phenolphthalein as indicator. The initial titration has been deducted from the subsequent figures so that the table represents actual increases in acidity. The mixtures were kept at room temperature and were subject to its slight fluctuations; all were shaken interchangeably and exactly alike. Knowing the weight of triolein in each digestion, the total available acid can be determined and thus the per cent of the ester hydrolyzed in each case. Every precaution was taken to secure uniformity in sampling and end points. A duplicate digestion of No. I made as a check. The duplicate

TABLE I.

NO.	I.	II.	III.	IV.	V.	VI.
1.	0.55	1.00	1.10	1.15	1.50	1.80
2.	1.20	1.45	1.65	2.20	2.70	2.70
3.	1.60	2.05	2.40	3.00	3.60	3.60
4.	1.80	2.80	3.40	3.40	4.45	5.15
5.	2.85	4.05	6.15	6.50	7.75	9.10
6.	3.30	4.75	6.50	7.65	8.50	10.70
7.	4.20	6.60	10.30	11.60	13.40	17.20
8.	4.30	8.00	12.20	15.50	17.95	22.25
9.	6.30	9.30	13.70	17.60	17.80	23.90
10.	7.00	9.70	14.70	19.10	20.10	25.60

results are so nearly identical that one may evidently rely on the uniformity of the surface exposed to the enzyme in the emulsion. The curves themselves are regular and thus furnish additional proof of the reliability of the procedure.

It will be seen that in this case an increase in the substrate upon increases the amount of acid liberated. But doubling the triolein does not double the oleic acid produced. As the amount of oil grows larger in the series the acidity at equilibrium increases

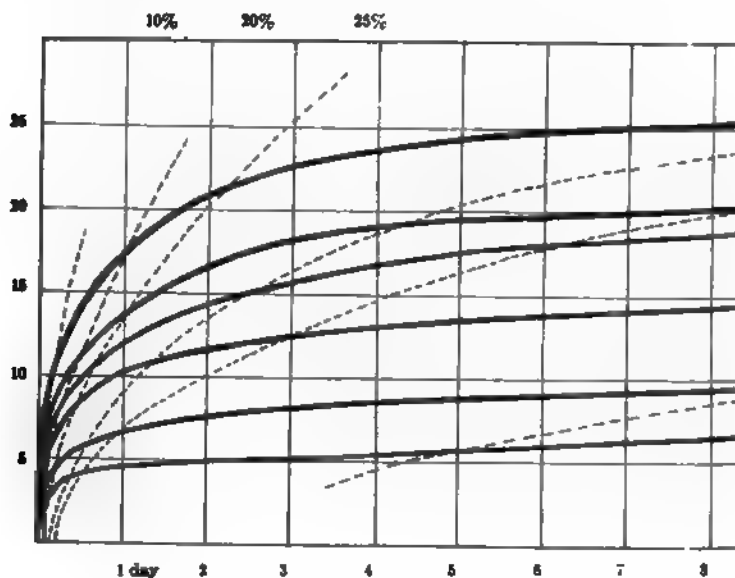


FIG. 1.

at a diminishing rate, suggesting that eventually a maximum amount of acid can be liberated by the addition of a very large amount of oil and that beyond that point further additions would produce no effect. This is indicated in another way by connecting the points at which a definite per cent of the oil is hydrolyzed. On the curves these are represented by dotted lines. At the very beginning of digestion the time required to hydrolyze two per cent of the oil is practically the same in all cases, but as higher percentages are chosen it is found that the time becomes longer in the larger digestions. At the beginning then, the

monomolecular, but as digestion proceeds there is a regular departure from monomolecular speed, and this departure is in the larger digestions. The amount of this slowing of reaction speed is evidently not attributable to the accumulating amount of the products, since the proportions are the same and it should be the same in all. It is an effect connected evidently with the amount of oil and its proportion to the enzyme decreasing amount of water. The dotted lines are apparent curves of a logarithmic type showing the regular effect of variable factor in the reaction. Water and oil are the only variables and the next experiment will show that water is in sufficient excess in these reactions to have a constant amount leaving oil as the only variable.

EXPERIMENT II. To determine within what limits water acts as a factor in such digestions as these three hydrolyses were made. Experiment I contained 10 cc. oil + 10 cc. juice; No. II, 10 cc. oil + 10 cc. juice + 10 cc. water; No. III, 10 cc. oil + 10 cc. juice + 80 cc. water. The three were subjected to the usual treatment of inter-shaking and sampling. The figures show that a 1:1 of water and oil is not sufficient for maximum hydrolysis under these circumstances. A ratio of 4:1 is however sufficient to give maximum results nor does an increase of the water to 9:1 alter the reaction speed or equilibrium. In Experiment I the ratio of water to oil in the largest digestion (f) is 9:1, so that there is always enough water enough present in all to give maximum results.

TABLE II.

TIME.	I.	II.	III.
a.....	0.40	1.70	2.70
b.....	1.40	3.60	4.40
c.....	2.30	5.50	5.80
d.....	3.10	5.90	6.90
e.....	4.50	7.20	7.90
f.....	7.90	17.70	17.00

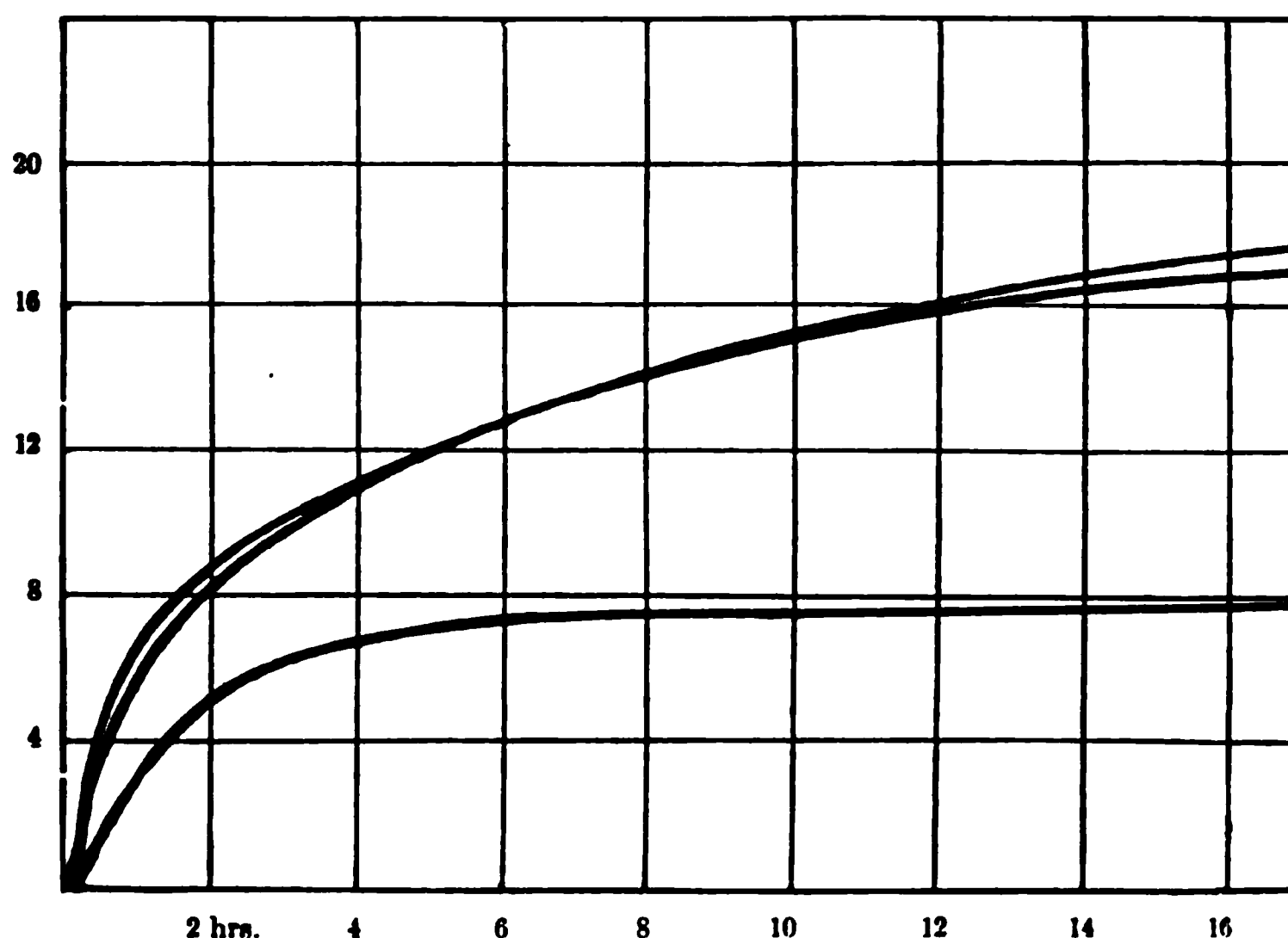


FIG. II.

**EXPERIMENT III.** Digestions were next made to determine effect upon final equilibrium of a regular increase of enzyme. Three series were started and allowed to stand from five to ten days, depending upon the amount of oil used. At the end of the digestion periods the acidity of each bottle was determined. The curves represent therefore points of equilibrium attained in reactions in the presence of varying amount of the enzyme. The ratios of water to oil in the three series were: 12:1, 6:1, and 3:1, respectively. Certainly in the first two and probably also in the third water was present in sufficient amount to function as a solvent. The same point is evident in all three, i.e., the more enzyme there is present in a digestion the more complete will the reaction be.

All three curves show digestion proportional to the square of the enzyme in the first portions. Thus in II and III increasing the lipase solution from 10cc. to 40cc. doubles digestion. The law of Schütze applies approximately at this point, but when the series is extended as in I it must be evident that its application is but a passing phase of the process. By selecting particular points along the curve one can show that digestion is proportional to the square root of the enzyme. It is certain that the relation is not a linear one and hence it is equally certain that a definite combination

TABLE III.

SERIES	NO.	JUICE.	OIL.	FINAL VOL.	$\frac{N}{10}$ ALK.	HYDROLYSIS
		cc.	cc.	cc.	cc.	per cent
{	1	1	2	42	28.40	25.0
	2	2	2	42	37.00	32.5
	3	3	2	42	42.3	37.00
	4	4	2	42	46.10	40.0
	5	5	2	42	48.75	42.0
	6	8	2	42	58.00	51.0
	7	10	2	42	61.00	54.0
	8	15	2	42	68.00	60.0
	9	20	2	42	78.00	68.5
	10	30	2	42	78.50	69.0
	11	40	2	42	86.00	76.0
{	12	1	10	60	31.2	5.0
	13	10	10	60	180.50	31.5
	14	20	10	60	262.8	46.0
	15	30	10	60	320.0	56.0
	16	40	10	60	376.0	64.0
{	17	1	20	60	45.00	4.0
	18	10	20	60	270.00	24.0
	19	40	20	60	550.00	48.0

ne and zymolite, in the sense of a neutralization of a base by acid or the precipitation of a salt, does not take place. The results suggest rather a mass action of the enzyme upon the reactants similar to the mass effect of water in the hydrolysis of an unreactive ester or in the ionization of a poor electrolyte.

*pseudo-equilibria.* It has been generally assumed that the hydrolysis of an ester is brought to a standstill before the reaction has reached that equilibrium which the relative masses of water and products should bring about. These points of premature stoppage constitute pseudo-equilibria, and are thought to be due to the protective action of the free fatty acid upon lipase. If the lipase were not destroyed the reaction would go on to its true point of equilibrium, as it does when a strong acid is the catalyzer. It is believed also that the presence of neutral oil in the mixture exerts a protective action over the lipase, and this assumption seems all

the more plausible in view of the fact that where a large amount of oil is present much more acid will be set free by a given amount of enzyme than in a digestion where the oil is relatively small. Digestion does not proceed twice as far when twice the oil is present perhaps because of the longer contact between acid and lipase in the larger digestion. Thus in experiment I equilibrium is much later in (f) than in (a); the enzyme is thus exposed to a large amount of acid a longer time.

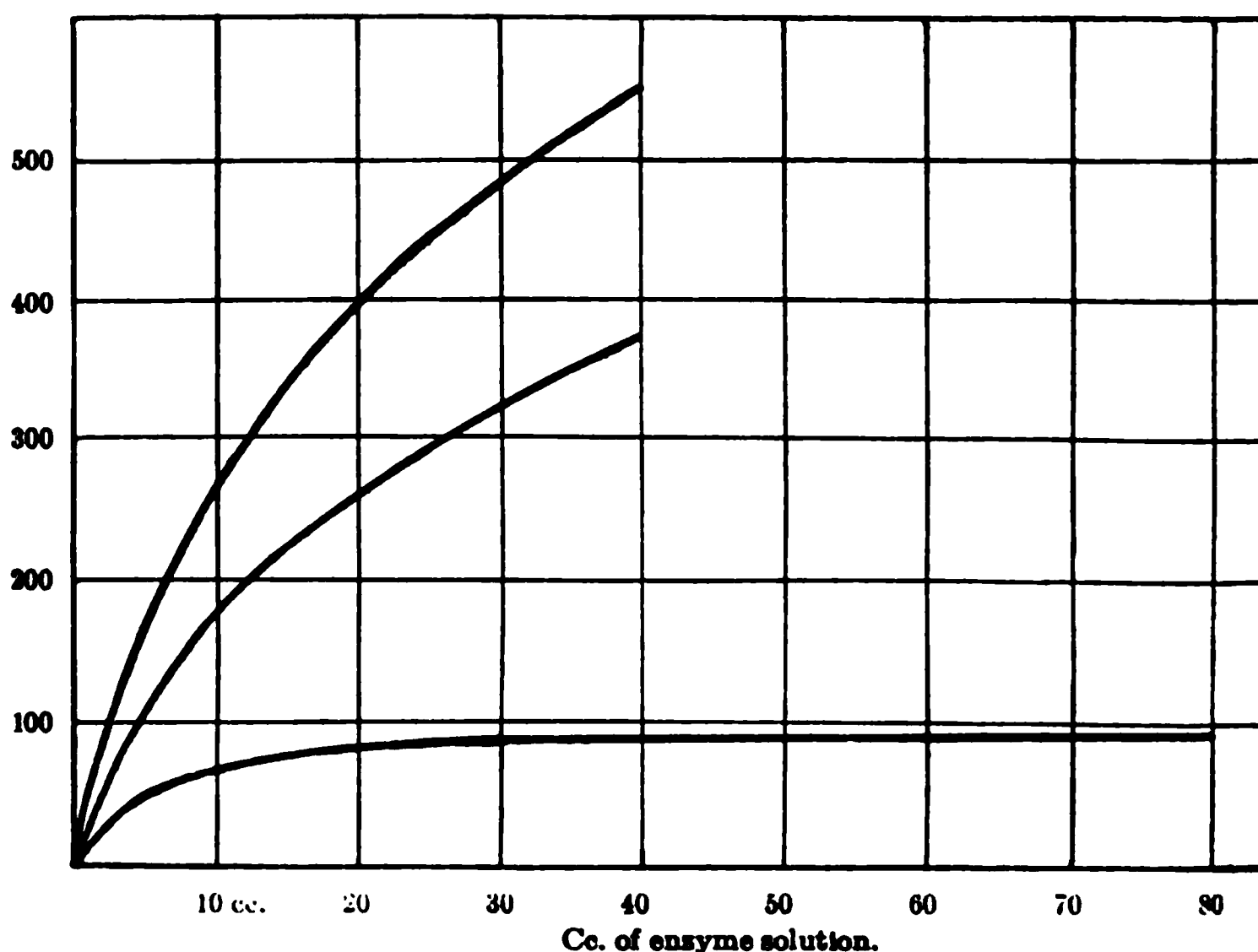


FIG. III.

Granting that the presence of the neutral ester may protect the lipase from the deleterious effect of acid, what is the probable mechanism of this action? Two possibilities occur to one. 1. The ester may simply dilute the acid; in the case of an insoluble acid like oleic practically all of the acid would be present dissolved in the neutral oil. Any inert fat solvent should then have the same protective action as an excess of neutral oil. Digestions have been carried out to test this hypothesis using an excess of ether and of chloroform. We have not been able to detect any effect upon the point of equilibrium by the addition of these substances.



Second, the ester may act like a neutral salt in the presence of its acid in decreasing the effective acidity of the solution. Since this is an ionic reaction it would apply only to the lower fatty acids and their esters where the ester and acid are soluble in water and the acid dissociated. In the case of oleic acid and triolein where solubility and ionization are extremely slight the hydrogen ion could have no significance. Furthermore oleic acid has not been shown to have a destructive action upon lipase so far as we have found. Undoubtedly the soluble and dissociable fatty acids like acetic acid do destroy lipase and the higher homologues may be expected to do the same in proportion to their solubility and ionization in water. Oleic acid is practically insoluble and non-dissociable in water. If it actually did destroy lipase that fact alone would render the theory of fat synthesis by reversibility of the reaction practically untenable. The recent experiments of Hamsik<sup>1</sup> are sufficient to show that oleic acid has little or no destructive action upon lipase. Intestinal and pancreatic powders were mixed with oleic acid, glycerine and a little water. At the end of a week a 28 per cent reversion had resulted. The same powder was then separated from the mixture, repurified with ether, and used in a second experiment. A week's contact with acid and glycerine gave a second synthesis of 19 per cent. The treatment with ether in repurifying the powder was sufficient to account for the lessened activity during the second week, so that we may assume that a week's contact with oleic acid and glycerine under extreme conditions did not injure the lipase to an appreciable extent. It is therefore quite improbable that in the hydrolyses described in this paper the pseudo-equilibria are due to destruction of lipase by oleic acid.

*Extent of Hydrolysis.* It remains to be shown to what extent digestion of triolein can be carried by sufficiently increasing the proportion of lipase to oil in the presence of an excess of water. We have collected in the following table the results of many hydrolyses reduced to a common unit of enzyme for the sake of comparison. Forty cc. of juice No. V was selected as the lipase unit.

To several of these hydrolyses the objection can be made that water is present in relatively small amount. In digestions 14, 20,

<sup>1</sup> A. Hamsik: *Zeitschr. f. physiol. Chem.*, lix.

TABLE IV.

NO.	JUICE.	OIL.	TOTAL VOL.	RATIO WATER: OIL.	RATIO JUICE: OIL.	ALK. $\frac{N}{10}$ .	DI
	cc.	cc.	cc.			cc.	1
1	40.0	0.5	42	80:1	40: 0.5	26.30	
2	40.0	1.0	42	40:1	40: 1.0	45.60	
3	20.0	0.5	60	120:1	40: 1.0	24.60	
4	20.0	1.0	60	60:1	40: 2.0	39.4	
5	40.0	2.0	42	21:1	40: 2.0	86.00	
6	20.0	2.0	60	30:1	40: 4.0	73.00	
7	20.0	2.0	42	21:1	40: 4.0	78.20	
8	20.0	4.0	60	15:1	40: 8.0	127.4	
9	10.0	2.0	42	21:1	40: 8.0	61.00	
10	20.0	5.0	60	12:1	40:10.0	155.4	
11	8.0	2.0	42	21:1	40:10.0	58.00	
12	20.0	8.0	60	7:1	40:16.0	242.80	
13	5.0	2.0	42	21:1	40:16.0	48.7	
14	40.0	20.0	60	3:1	40:20.0	550.00	
15	20.0	10.0	60	6:1	40:20.0	268.00	
16	4.0	2.0	42	21:1	40:20.0	46.10	
17	3.0	2.0	42	21:1	40:27.0	42.30	
18	20.0	15.0	60	4:1	40:30	360.00	
19	2.0	2.0	42	21:1	40:40	37.00	
20	20.0	30.0	80	2:1	40:60	430.00	
21	20.0	40.0	60	3:1	40:80	380.00	
22	10.0	20.0	42	2:1	40:80	270.00	
23	1.0	2.0	42	21:1	40:80	28.40	
24	5.0	20.0	42	2:1	40:160	120.70	
25	1.0	20.0	42	2:1	40:800	45.00	

21, 22, 24 and 25 water is below the ratio of 4:1 which we usually used to insure a maximum hydrolysis. On the other 23 would seem to indicate that even the ratio of 2:1 is capa giving maximum results. At least in the two cases 22 and have the same result though with a ratio of water to oil of : one case, 21:1 in the other.

While the points of equilibrium do not fall on the same line indicate fairly well that the direction of the curve is about as d The extrapolation from 93 per cent to 100 per cent seems als tifiable from the general trend of the curve. Digestion is con under these conditions when the ratio of enzyme to oil is ;

Within the range of conditions prevailing in the intestine during digestion it is evident that hydrolysis would tend to approximate completion if given sufficient time. With the products removed and fresh enzyme continually added to the digesting mixture it would probably approximate total hydrolysis during the normal period of intestinal digestion.

Curve b represents the actual amount of acid liberated by 40 cc. of the given juice with different amounts of oil. The curve is drawn as cc. of  $\frac{N}{10}$  acid as indicated by the column of figures to

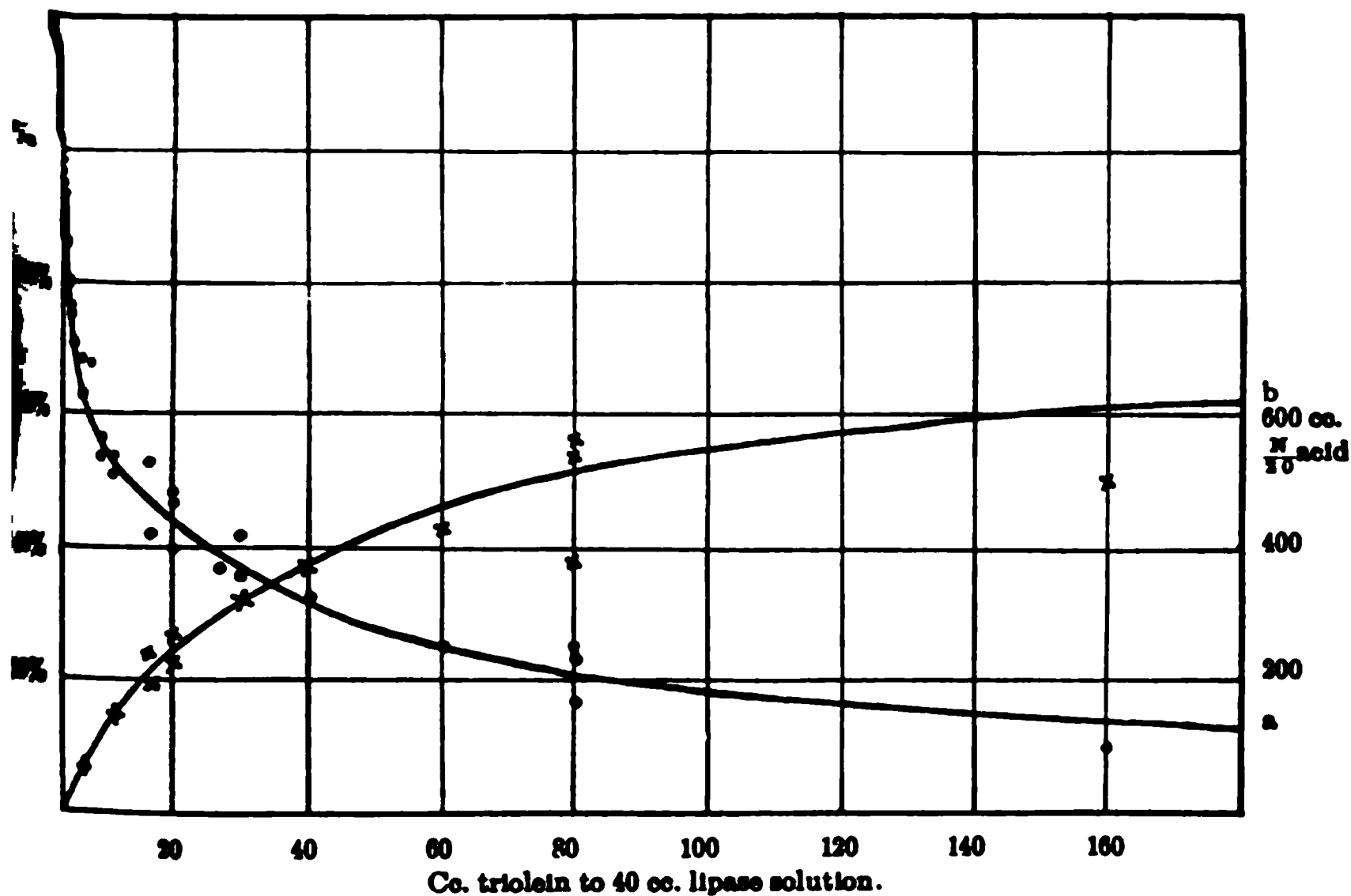


FIG. IV.

the right. As the amount of oil becomes large the production of acid reaches a maximum point beyond which further increases in the oil present leads to little or no increase in total acid developed. This curve therefore confirms the suggestion of such a maximum point mentioned in connection with the first experiment.

The facts collected above seem to indicate a real difference between lipase and a true catalytic agent. The latter does not alter the point of equilibrium nor the direction of the reaction. Lipase apparently exerts a mass effect upon the reaction so that

hydrolysis may be carried from 0 per cent to 100 per cent by varying the mass. While analogies are often misleading, the analogy suggested earlier in the paper is striking enough to deserve further amplification. If we consider the case of a slightly ionizable salt, base or acid in the presence of varying masses of water, we find the curves of hydrolysis as measured by conductivity to be practically identical with the type of curves obtained from the lipase reactions. Thus curve III represents the dissociation of acetic acid or ammonia in the presence of increasing amounts of water. As is well known dissociation is roughly proportional to the square root of the water present; the law of Schütze is an approximate statement of fact during a stage of the process, as it is in lipase reactions. When dilution becomes very great dissociation is practically complete so that further dilution does not appreciably increase the conductivity and the square root expression no longer applies. Again if a given volume of water is taken and a weak electrolyte added to it we get the same type of curve as in IV. When a minute amount of the salt is present dissociation is practically complete. As more salt is added the actual number of ions will increase while the percentage dissociation falls. A point is reached where the given volume of water will dissociate no more of the salt—the solution becomes saturated with ions and the additions of the salt have no further effect upon the number. It is not desired to emphasize this analogy except to bring out the point that in the process of hydrolysis and dissociation of a salt in water we have a reaction which is typically controlled by mass action. In the action of lipase upon triolein we find results that suggest a similar mass effect, and if this is the fact it constitutes a difference between enzymes and the inorganic, soluble catalytic agents. This action of the enzymes has been explained as due to the fact that the reacting mixture is heterogeneous—the enzyme is in colloidal solution. It is not clear just why the change from a soluble ester to an emulsion of an insoluble one does not also show some marked effect upon the reaction. Nor is it clear why the enzyme, active at the beginning of the reaction and undestroyed at the end, should fail to facilitate the hydrolysis right through to the point of theoretical equilibrium as determined by the masses of water, ester and hydrolytic products. It is not clear why the products of the reaction—if stoppage is to be referred to the accumulation of products—should influence a colloidal

catalytic agent any differently than a soluble one. If the peculiarities of such enzyme reactions are due to the heterogeneity of the mixture then doubling the enzyme should double the effect, but this we have shown is not the case.

*Reversibility.* Whether the peculiarities of the enzyme catalysis can be completely explained on the basis of a heterogeneous reaction or not, the results presented here have a definite bearing upon reversibility and fat synthesis in the body. In view of the data, one may confidently assert that under the conditions which obtained in these experiments reversion cannot take place to an appreciable extent. Under such conditions of excess of water and pancreatic lipase the reaction is irreversible. Nevertheless we have recently had several successful examples of reversion reported in the literature. Pottevin<sup>1</sup> obtained considerable amounts of mono-olein and triolein by a reversion experiment. Hamsik reported as much as 30 per cent reversion to triolein in some cases. The significant point in the work of both these investigators is the absence of water from the reacting mixtures. Dry pancreas and intestinal powders were used as a source of lipase, mixed with pure glycerin and oleic acid. In some cases Hamsik added an insignificant amount of water. Whenever water in significant amounts was present the synthesis failed to occur, and when water was added to the synthesizing mixture hydrolysis at once began and hydrolytic products alone remained at the end of the experiment. If water is present to the extent of 50 per cent of the total mixture the synthetic process cannot be detected. Our own experiments practically confirm this point. In Curve II, 50 per cent of water was not sufficient to give maximum hydrolysis and we may assume therefore that a certain amount of synthesis could take place. In Table IV, however, it is seen that 66 per cent of water does give maximum hydrolysis. It is probable that a slight variation in the water present when it approximates 50 per cent of the mixture modifies considerably the resultant reaction, and between 50 and 66 per cent a point is reached where hydrolysis is so much more rapid than synthesis that the latter becomes a negligible quantity.

In assuming therefore that lipase plays an important part in

<sup>1</sup> Henri Pottevin: *Comptes rend.* cxxxci, p. 378.

the building up of fat in the cells of the body one must points of practical dessication in the protoplasm where radicals come in contact with lipase and fatty acids. Such condition is of course not inconceivable though we believe little has been taken of the fact in formulating the theories of fat action and synthesis. It perhaps makes the reversion hypothesis a little less acceptable as an explanation of fat-storage. It is possible that the production of fat from carbohydrates does not involve the action of lipase at all, and that in general fat synthesis is brought about by condensation reactions other than the action of the hydrolytic cleavage. The function of lipase may well be hydrolytic only in the body, though capable of accelerating the reverse action when placed under the extreme conditions of laboratory demonstration.

#### SUMMARY.

1. In the presence of an excess of water the hydrolysis of triolein is regularly increased by an increased amount of lipase present. The rate of increase suggests a mass effect of lipase on the equilibrium of the reaction. By sufficiently increasing the amount of lipase the reaction may be carried to completion.

2. A given amount of lipase can, under optimum conditions, liberate a definite amount of fatty acid from triolein, irrespective of the mass of the latter.

3. When water is present to the extent of more than 50 per cent of the mixture, reversion is negligible and the reaction proceeds toward the complete hydrolysis of the ester. Reversion becomes appreciable under conditions of concentration so high as to approach dessication.

4. It is possible that lipase, while important in the hydrolysis and absorption of fats, is not important as a factor in the synthesis and storage in the cell.

# BEHAVIOR OF MOLDS TOWARD THE STEREO-ISOMERS OF UNSATURATED DIBASIC ACIDS.

By ARTHUR W. DOX.

(From the Storrs Agricultural Experiment Station.)

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In a brief paper entitled "Notiz aus der Gärungschemie"<sup>1</sup> Buchner in 1892 described some experiments in which he attempted to cultivate molds upon the ammonium salts of fumaric and maleic acids. The medium he used consisted of a solution of the acid in the form of its neutral or acid ammonium salt, with potassium phosphate, magnesium sulphate and calcium chloride for the inorganic constituents. The organisms tested were *Penicillium glaucum* and *Aspergillus niger*. Buchner found that these molds could utilize fumaric but not maleic acid as a source of carbon. This he was led to expect from the fact that fumaric acid is a normal constituent of many plants, while maleic acid has not yet been found in nature. The distinction was surprising, however, because of the readiness with which these two acids undergo molecular rearrangement and can be converted into each other.

As Buchner's experiment was made with only two species of mold, the writer thought it would be of interest to repeat the experiment with a larger number of organisms and include also the homologues of these two acids, viz., mesaconic (methyl fumaric) and citraconic (methyl maleic). The isomeric itaconic acid (methylene succinic) was also included. These acids were obtained from Merck & Co., and were found to have the correct melting points.

Solutions were prepared containing fifth-molecular amounts of the acid ammonium salts of these acids, and the inorganic

<sup>1</sup>*Ber. d. deutsch. chem. Gesellsch.*, xxv, pp. 1161-1163.

constituents were added as follows,—potassium acid phosphate 0.1 per cent, magnesium sulphate 0.05 per cent, sodium chloride 0.1 per cent. The molds were grown in test tubes containing 5 cc. of the medium. In the first experiment the medium was sterilized in an autoclave at 110°. As the molds grew equally well upon the fumaric and maleic acids in this case, it was suspected that molecular rearrangement might have taken place during sterilization, and such was found to be actually the case. No growth, however, had occurred on the other three acids at the end of three weeks.

In the second experiment the medium was prepared under conditions as nearly aseptic as possible and transferred to test tubes. Before inoculation the tubes were allowed to stand for 24 hours and as no growth of any kind had appeared at the end of that time they were considered practically sterile. Inoculations were made with spores of mold taken from pure cultures. The tubes were kept in a thermostat at 20 degrees and examined at the end of three weeks. The results are given in the following table. 0 indicates germination only, + slight growth, ++ fair growth, +++ good normal culture, and 0 no growth at all.

	FUMARIC	MALEIC	MESACONIC	CITRACONIC	ITACONIC
<b>Penicillium</b>					
camemberti.....	+++	G	0	0	
roqueforti.....	+	G	0	0	
expansum.....	+++	G	0	0	
chrysogenum.....	+++	G	0	0	
purpurogenum....	+	+	0	0	
<b>Aspergillus</b>					
niger.....	+	+	0	0	
clavatus.....	++	+	0	0	
ochraceus.....	+++	+	0	0	
candidus.....	+	0	0	0	
flavus.....	++	+	0	0	

While maleic acid gives in some cases a scanty growth, it can hardly be regarded as a source of carbon for any of these molds. Fumaric acid, on the other hand, is quite readily utilized.



trary to what might be expected, neither mesaconic nor citraconic acids could be utilized. These acids bear exactly the same relation to each other as regards solubility, configuration and anhydride formation, as do fumaric and maleic, yet neither is available by the molds as a source of carbon. Itaconic acid gave in some instances a slight growth, but no normal colony.



# THE PARTIAL HYDROLYSIS OF PROTEINS.

## II. ON FIBRIN-HETEROALBUMOSE.

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The study of partial cleavage has proved of great service for the interpretation of the constitution of many complex substances. The value of the same method for the elucidation of the structure of the protein molecule has been discussed by one of us some time ago.<sup>1</sup> Here, again, we wish to emphasize that we are fully aware of the difficulties which one encounters when attempting to separate the individual products of partial hydrolysis of proteins, both of colloidal and crystalline nature. However, we feel confident that the classical methods of the schools of Kühne, of Chittenden, of Hofmeister, and the recently added method of Siegfried and his co-workers—though from a chemical standpoint imperfect—still remain of great service for the study of the chemical structure of the protein molecule. The different albumoses may not represent chemical individuals; but even if they are mixtures, they are mixtures similar within each fraction not only by the physical properties, but also by the chemical composition of the parts of the mixture. This can be stated with certainty in regard to the so-called primary digestion products, the hetero- and the proto-albumoses. The work of Adler<sup>2</sup> and of Birchard<sup>3</sup> in Siegfried's laboratory has contributed much evidence in support of this view. The work undertaken in this laboratory several years ago, and which was planned to be a systematic study of every individual fibrin-albumose, and of the products of partial hydrolysis of the

<sup>1</sup> P. A. Levene, this *Journal*, i.

<sup>2</sup> Dissertation, Leipzig, 1907.

<sup>3</sup> Dissertation, Leipzig, 1909.

same, was for a time interrupted. This interruption was caused principally by work on the improvement in the methods of analysis of the products of protein hydrolysis. Considerable advance in the methods of analysis have been made through the efforts of Osbourne and his co-workers, of Siegfried and his pupils, and through the work done in this laboratory.

It was therefore concluded to resume the work on the products of partial hydrolysis of fibrin. The aim of the work is to find evidence which will lead to a definite solution of the following queries.

First, whether or not on partial hydrolysis the protein molecule is decomposed into large fragments—which appear in the form of albumoses.

Second, whether or not the partial hydrolysis of proteins proceeds by degrees, in a manner such that only individual amino-acids, or simple peptides are detached from the original molecule, so that by degrees the number of amino-acids contained in it decreases, and the protein is transformed through the more complex albumoses into the simpler, and finally into peptones, peptides, and amino-acids.

Third, whether or not the two processes occur simultaneously. In that case the task will arise to trace the relationship of the simpler albumoses or peptones to one or the other of the more complex substances.

The present communication is limited to the work on the preparation and on the hydrolysis of the hetero-albumose.

#### PREPARATION OF THE HETERO-ALBUMOSE.

The method of preparation consisted in a combination of each of the methods as worked out by Kühne and Pick, and was the same as that adopted by one of us<sup>1</sup> in a former investigation.

The details of the method are as follows:

A 10 per cent solution of Witte's peptone was carefully neutralized with dilute sulphuric acid, allowed to stand over night, and the undissolved residue filtered off. It was later found that the solution could be centrifugalized

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<sup>1</sup> P. A. Levene: this *Journal*, i, p. 1.

advantage and consequent saving of considerable time. To the clear solution obtained by either method was added an equal volume of a concentrated solution of ammonium sulphate. The primary albumoses were filtered off, washed with half-saturated ammonium sulphate solution, and were reprecipitated in half the former dilution. The hetero- was separated from the proto-albumose by adding an equal volume of 95 per cent alcohol, allowing to stand two days, and filtering off the hetero-albumose by the aid of a filter. The product so obtained was carefully washed with 50 per cent alcohol, dissolved in a volume of warm water equal to half of that formerly employed, and twice reprecipitated by the addition of an equal volume of 95 per cent alcohol. The final product was dissolved in warm water, a little ammonium sulphate being added to aid the solution. The product was then subjected to dialysis until no more sulphate could be detected by barium chloride. By this process a fine granular substance was obtained which was repeatedly washed by decantation in a large excess of distilled water. This method involves a very large loss of material, but it was only in this way that a pure product could be obtained.

### *Properties.*

The albumose obtained in this way was extremely insoluble in water; when suspended in 4 liters of water and after intermittent stirring for 12 hours, 100 cc. of the supernatant liquid contained only 0.0042 gm. of nitrogen or 0.025 gm. of albumose. For analysis a sample was dried by heating to constant weight under diminished pressure at the temperature of boiling water. The following results were obtained:

0.2056 gm. substance gave 0.3734 gm.  $\text{CO}_2$  and 0.1220 gm.  $\text{H}_2\text{O}$ .

0.1628 gm. of the substance employed for a nitrogen estimation after Kjeldahl, required 19.15 cc.  $\frac{N}{10}$   $\text{H}_2\text{SO}_4$ .

These results agree fairly well with those obtained by Adler<sup>1</sup> with Levene's hetero-albumose, as can be seen from the following table:

<i>Adler's Analysis.</i>		<i>Present Analysis.</i>	
	<i>Per Cent</i>		<i>Per Cent</i>
C =	48.18	C =	49.52
H =	6.63	H =	6.64
N =	16.00	N =	16.46

For further identification the rotatory power of the substance dissolved in 5 per cent ammonium sulphate solution was deter-

<sup>1</sup> Dissertation, Leipzig, 1907.

mined. For this determination very careful drying is necessary, as prolonged heating at temperatures over  $100^{\circ}\text{C}$ . tends to change the substance into a form insoluble even in salt solution. The albumose was first dried two days under diminished pressure over sulphuric acid, then for two days under diminished pressure at the temperature of boiling chloroform, and finally to constant weight at the temperature of boiling alcohol.

0.1615 gm. substance, dissolved in 25 cc. rotated  $-0.84^{\circ}$  in 1.855 gm. tube at  $t = 20^{\circ}\text{C}$ . Thus:

$$[\alpha]_D^{20} = -70.11^{\circ}.$$

This is in close agreement with the value found by Adler,  $-70.69^{\circ}$  for an ammoniacal solution of the hetero-albumose.

*Primary Amino Nitrogen:* 0.2393 gm. albumose treated with nitrous acid gave 4.40 cc. of nitrogen gas at  $21^{\circ}$ , 756 mm.

Amino N = 1.03 per cent = 6.3 per cent of the total N.

#### HYDROLYSIS.

##### *Glutaminic Acid and Esterified Acids.*

One-hundred and twenty-nine grams of the hetero-albumose were hydrolyzed by boiling 15 hours, with 20 per cent hydrochloric acid. The solution was concentrated, saturated with hydrochloric acid, and left for 10 days in the refrigerator. The glutaminic hydrochloride was filtered on asbestos and recrystallized. 10.83 gms. of the pure hydrochloride, equivalent to 5.67 gms. of glutaminic acid, were obtained.

*Analysis:* 0.4370 gm. subst.; 23.80 cc.  $\frac{N}{10}$  Ag NO<sub>3</sub>.

	Calculated for C <sub>6</sub> H <sub>9</sub> O <sub>4</sub> N.HCl:	Found:
Cl.....	19.31 per cent	19.32 per cent

The mother liquors were esterified according to Fischer's method, three crops of esters being freed with barium hydrate and extracted by the method of Levene and Van Slyke.<sup>1</sup> The esters were distilled, using H<sub>2</sub>SO<sub>4</sub> in place of liquid air refrigeration, to absorb uncondensed vapors.<sup>2</sup> The esters boiling above  $90^{\circ}$  were not

<sup>1</sup> Levene and Van Slyke: this *Journal*, vi, p. 391. 1909.

<sup>2</sup> Levene and Van Slyke: *Biochem. Zeitschr.*, x, p. 214, 1908.

distilled, but worked up as recently proposed by Osborne and Jones.<sup>1</sup> The results were satisfactory.

The esters were divided into the following fractions by distillation.

	TEMPERATURE OF VAPORS.	PRESSURE.	WEIGHT OF ESTERS.
	degrees	mm.	gm.
I.....	to 60	12.0	27.8
II.....	to 90	0.4	32.0
III.....	Undistilled	....	32.3
Total .....			92.1

*Fraction I* was chiefly alcohol. It yielded by crystallization 0.95 gm. of a mixture containing 14.18 per cent N, and on evaporating the mother liquors to dryness, 0.96 gm. of more soluble acids. The first crop was worked up with the alanin-valin mixtures from *Fraction II*. The second crop was combined with the more soluble portion of *Fraction II*, and extracted with absolute alcohol to remove prolin.

#### *Leucin-Valin Sub-Fraction.*

*Fraction II* yielded three fractions by crystallization, which nitrogen determinations by the nitrous acid method<sup>2</sup> showed to be mixtures of valin and leucin, the weights and nitrogen contents being 2.96 gms., 11.07 per cent N; 4.00 gms., 11.34 per cent N; 4.12 gms., 11.71 per cent N. These fractions were combined, and the leucin, isoleucin and valin determined by precipitating the leucin isomers as lead salts, and determining the proportion of the two isomers by the rotation of their mixture in 20 per cent HCl.<sup>3</sup> One-half of the mixture was used for the lead separation. It yielded 6.91 gms. of the lead salt of leucin, equivalent to 7.76 gms. of the leucin isomers from the entire mixture.

<sup>1</sup> *Amer. Journ. of Physiol.*, xxvi, p. 212, 1910.

<sup>2</sup> D. D. Van Slyke: Method for Determination of Amino Nitrogen. *Proceedings Soc. Exp. Biol. and Med.*, December 15, 1909; *Ber. d. d. chem. Ges.*, xliii.

<sup>3</sup> Levene and Van Slyke: Analysis of the Leucin Fraction of Proteins, *this Journal*, vi, p. 391, 1909.

**Analysis:** 0.2958 gm. subst.; 0.1924 gm.  $\text{PbSO}_4$ :  
0.2158 gm. subst.; 22.5 cc. N at  $18^\circ$ , 756 mm. (nitro  
method).

	Calculated for $\text{Pb}(\text{C}_6\text{H}_{13}\text{O}_2\text{N})_2$ :	Found:
Pb.....	44.29 per cent	44.32 per cent
N.....	6.00 per cent	5.92 per cent

The leucin and isoleucin were freed from their salts as described by Levene and Van Slyke, and the rotation of the mixture in 20 per cent HCl.

0.2632 gm. subst; 5.016 gm. solution; concentration, 5.15 per cent  
tion in 1 dm. tube,  $+1.52^\circ$

$$[\alpha]_D^{20} = +26.32^\circ.$$

Calculated from the rotation, the mixture contained 50 cent of *l*-leucin, 49.2 *d*-isoleucin, or 3.94 and 3.82 gms. respectively. The mixture of the free leucin isomers gave the following results on analysis:

0.1570 gm. subst.; 30.34 cc. N at  $24^\circ$ , 760 mm. (nitrous acid method)

	Calculated for $\text{C}_6\text{H}_{11}\text{O}_2\text{N}$ :	Found:
N.....	10.69 per cent	10.78 per cent

The filtrate from the leucin lead salts yielded 1.46 gm. valin, equivalent to 2.92 gms. for the entire portion.

**Analysis:** 0.1141 gm. subst.; 25.30 cc. N at  $30^\circ$ , 756 mm. (nitrous acid method).

0.1594 gm. subst.; 0.3082 gm.  $\text{CO}_2$ ; 0.1490 gm.  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_5\text{H}_{11}\text{NO}_2$ :	Found:
N.....	11.96 per cent	11.88 per cent
C.....	51.24 per cent	51.33 per cent
H.....	9.47 per cent	9.64 per cent

### *Valin-Alanin Sub-Fraction.*

The mother liquors from the above crystallization fraction were freed from prolin by extraction in the usual manner with absolute alcohol, then submitted to fractional crystallization. 1.65



more of valin was obtained, making the entire yield of pure valin 4.57 gms.

*Analysis:* 0.1340 gm. subst.; 30.5 cc. N at 32°, 756 mm. (nitrous acid method).

	Calculated for $C_5H_{11}O_2N$ :	Found:
N.....	11.96 per cent	12.08 per cent.

Rotation in 20 per cent HCl: 0.2460 gm. substance; 5.114 gm. solution; concentration, 4.81 per cent; rotation in 1 dm. tube, + 1.25.°

$$[\alpha]_D^{20} = + 23.63^\circ.$$

3.61 gms. of recrystallized alanin were obtained from the valin mother liquors.

*Analysis:* 0.1161 gm. subst.; 34.7 cc. N at 32°, 756 mm.  
0.1388 gm. subst.; 0.2054 gm. CO<sub>2</sub>; 0.0955 gm. H<sub>2</sub>O.

	Calculated for $C_3H_7O_2N$ :	Found:
N.....	15.73 per cent	15.85 per cent
C.....	40.42 per cent	40.36 per cent
H.....	7.93 per cent	7.69 per cent

1.10 gms. of a mixture of alanin and valin (C = 44.81, H = 8.58) were obtained which could not be separated. No glycocoll could be obtained by the carbamino method of Siegfried<sup>1</sup> although this method was tried out on mixtures of glycocoll and *d*-alanin and found to be fully as satisfactory as Siegfried claims. For example from a mixture of 0.37 gm. glycocoll and 1.00 gm. *d*-alanin in 50 cc. baryta water, 0.28 gm. of pure glycocoll was obtained (N = 18.90, calc. 18.67). The failure to detect glycocoll by this method therefore indicates that very little if any was present. No fractions which from analysis appeared to be mixtures containing glycocoll could be obtained on recrystallization.

### *Prolin.*

The amino-acids completely soluble in cold alcohol were dissolved to 100 cc. in water, and the prolin determined by the method of Van Slyke.<sup>2</sup> Kjeldahls on 5 cc. samples required 35.75 — 35.65

<sup>1</sup> Ber. d. d. chem. Ges., xxxiv, p. 400.

<sup>2</sup> Ber. d. d. chem. Ges., xliii.

cc. of  $\frac{N}{10}$   $\text{H}_2\text{SO}_4$  for titration, indicating 1.000 gm. of nitrogen in the entire solution. Determination of the primary amino nitrogen in 10 cc. of the solution gave 71.10 cc. of nitrogen gas at  $21^\circ$ , 762 mm. indicating that 0.4025 gm. of the nitrogen came from the ordinary aliphatic amino-acids, 0.5975 gm. from the prolin in the mixture. This corresponds to 4.92 gms. of prolin.

The 80 cc. of the solution, remaining after removal of the sample for the above determinations, was racemicized, boiled with  $\text{CuC}$  and the prolin obtained as crystalline *d-l* copper salt. 3.77 gms. were regained, equivalent to 60.6 per cent of the amount of prolin calculated.

*Analysis:* 0.3784 gm. subst.; loss at  $100^\circ$ , 0.0385 gm.; 11.70 cc. ammonium sulphocyanide (Volhard titration),  
0.2310 gm. subst.; amino nitrogen, 4.70 cc. at  $19^\circ$ , 760 mm

	Calculated for $\text{Cu}(\text{C}_5\text{H}_5\text{O}_2\text{N})_2 \cdot 2\text{H}_2\text{O}$ :	Found:
$\text{H}_2\text{O}$ .....	10.99 per cent	10.72 per cent
$\text{Cu}$ .....	19.41 per cent	19.72 per cent
Amino N.....	0.00 per cent	1.16 per cent

After recrystallizing three times the prolin salt was obtained almost free from amino nitrogen. The amino nitrogen test is by far the most delicate for the purity of prolin obtained from proteins.

*Analysis:* 0.3484 gm. subst.; 0.0375 loss at  $100^\circ$ ; 10.70 cc.  $\frac{N}{10}$  ammonium sulphocyanide. 0.1906 gm. subst.; 0.60 cc. N at  $30^\circ$ , 760 mm. (nitrous acid method).

	Calculated for $\text{Cu}(\text{C}_5\text{H}_5\text{O}_2\text{N})_2 \cdot 2\text{H}_2\text{O}$ :	Found:
$\text{H}_2\text{O}$ .....	10.99 per cent	10.76 per cent
$\text{Cu}$ .....	19.40 per cent	19.51 per cent
Amino N.....	0.00 per cent	0.17 per cent

The mother liquors from the first crop of copper salt were free from copper by hydrogen sulphide, and the amino-acids crystallize from dilute alcohol. 1.35 gms. were thus obtained, a mixture of alanin and valin ( $\text{N} = 13.72$  per cent). The mother liquor were reconverted into copper salts, and yielded 0.57 gm. more of prolin salt ( $\text{H}_2\text{O} = 11.04$ ,  $\text{Cu} = 19.62$ , amino N = 0.72), making the total crystallized copper salt regained 69.7 per cent of the calculated by the nitrogen determinations.

*Phenylalanin.*

*Fraction III* of the esters was dissolved in water and the phenylalanin ester extracted with ether in the usual manner. The etheral extract contained practically all of the coloring matter. It was decolorized with charcoal, and the phenylalanin obtained as hydrochloride from aqueous solution after saturation with HCl. 3.85 gms. of the hydrochloride, equivalent to 3.16 gms. of phenylalanin, were obtained.

*Analysis:* 0.3201 gm. subst.; 16.05 cc.  $\frac{N}{10}$  silver nitrate.

	Calculated for $C_9H_{11}O_2N$ :	Found;
Cl.....	17.58 per cent	17.76 per cent

*Glutaminic and Aspartic Acids from Esters.*

The esters not extracted from water solution by ether were hydrolyzed with barium hydrate, as usual, the solution was freed from barium with sulphuric acid, and the glutaminic acid crystallized as hydrochloride. 4.47 gms. equivalent to 3.59 gms. of glutaminic acid were obtained.

*Analysis:* 0.2970 gm. subst.; 16.30 cc.  $\frac{N}{10}$  silver nitrate.

0.1430 gm. subst.; 19.80 cc. N at 25°, 760 mm. (nitrous acid method).

	Calculated for $C_6H_9O_4N.HCl$ :	Found:
Cl.....	19.31 per cent	19.45 per cent
N.....	7.63 per cent	7.68 per cent

The mother liquors were freed from HCl by concentration *in vacuo*, followed by use of silver sulphate, hydrogen sulphide, and an equivalent of barium hydrate to remove  $SO_4$ . The solution was concentrated and mixed with several volumes of alcohol. 6.10 gms. of aspartic acid crystallized on standing in the refrigerator.

*Analysis:* 0.1297 gm. subst.; 22.30 cc. N at 29°, 758 mm. (nitrous acid method).

0.1272 gm. subst.; 0.1643 gm.  $CO_2$ ; 0.0614 gm.  $H_2O$ .

	Calculated for $C_4H_7O_4N$ :	Found:
N.....	10.54 per cent	10.58 per cent
C.....	36.06 per cent	36.28 per cent
H.....	5.30 per cent	5.40 per cent

No serin could be obtained from the mother liquors.

*Prolin, Alanin, and Glycocoll from Unextracted Ester Residue*

The barium residues left after the third extraction of es were freed from hexone bases by phosphotungstic acid, and from sulphate, chloride, and excess phosphotungstate by barium hydroxide and silver sulphate. The solution of amino-acids was concentrated and 1 gm. of tyrosin filtered off. The other amino-acids were converted into copper salts; the salts proved extremely soluble in water. Their solution was brought to 150 cc. and 600 cc. of alcohol added. The precipitated salts dried at 100° *in vacuo* weighed 15.6 gms.; the salts soluble in 80 per cent alcohol, 8.5 gms.

The insoluble fraction was changed back to free acids. It refused to be crystallized from water or dilute alcohol. An attempt was made to obtain the picrate of glycocoll by Levene's method.<sup>1</sup> However, in place of the usual glycocoll picrate 1 gm. of a picrate differing in composition and in properties from the glycocoll compound were obtained. It was attempted to purify the substance by recrystallization from an alcoholic solution of picric acid. A great part of the substance remained in solution as only 0.4 gm. was obtained on recrystallization. This picrate was not explosive and contained only traces of mineral impurities. Tested in the usual manner for pyrrol, it gave a very definite positive reaction, melted between 235–240° C. (corr.) with decomposition and evolution of gas.

**Analysis:** 0.1248 gm. subst.; 15.7 cc. N (over 50 per cent KOH) at 763 mm.

	Calculated for $C_7H_5NO_2 \cdot C_6H_5(NO_2)_3OH$ :	Found:
N.....	18.42 per cent	14.28 per cent

Thus the substance was not glycocoll picrate. Lack of material did not permit of a detailed study of the substance at the present moment.

In the mother liquors of the first picrate on standing a second precipitate formed, about 0.80 gm. in weight with the properties of the glycocoll compound. M.p. = 190° C. (corr.) sharp.

<sup>1</sup> P. A. Levene, this *Journal*, i. p. 463.

*Analysis:* 0.120 gm. subst.; 20.0 cc. N (over 50 per cent KOH) at 30°, 757 mm.).

	Calculated for $C_6H_5NO_2.C_6H_5(NO_2)_3OH$ :	Found:
N.....	18.42 per cent	18.80 per cent

The copper salts soluble in 80 per cent alcohol were extracted with absolute alcohol. The soluble salts were reconverted into amino-acids, racemicized, changed back to Cu salts, and recrystallized from water. 0.75 gm. of anhydrous prolin copper salt, equivalent to 0.59 gm. prolin, was obtained. The product was not entirely pure, but was of the characteristic violet color when dried at 100°, and gave the following analysis.

0.3490 gm. subst.; 11.10 cc.  $\frac{N}{10}$  ammonium sulphocyanide.  
0.2200 gm. subst.; 9.30 cc. N at 32°, 756 mm. (nitrous acid method).

	Calculated for $Cu(C_6H_5O_2N)_2$ :	Found:
Cu.....	19.40 per cent	20.21 per cent
Amino N.....	0.00 per cent	2.15 per cent

The copper salts soluble in 80 per cent alcohol, but insoluble in absolute, were decomposed with  $H_2S$ , and the amino-acids crystallized from dilute alcohol in the hope of obtaining serin. Instead of serin, however, 0.75 gm. of alanin was obtained.

*Analysis:* 0.1339 gm. subst.; 0.0958 gm.  $H_2O$ .

	Calculated for $C_3H_7O_2N$ :	Found:
C.....	40.42 per cent	40.23 per cent
H.....	7.93 per cent	8.00 per cent

### *Tyrosin.*

Thirty-one grams of hetero-albumose were hydrolyzed with 20 per cent hydrochloric acid. The acid was removed by concentration *in vacuo* and the use of silver sulphate. The solution of amino-acids was concentrated *in vacuo* until crystallization began. 1.025 gms. of tyrosin were obtained.

*Analysis:* 0.1586 gm. subst.; 0.3475 gm.  $CO_2$ ; 0.0881 gm.  $H_2O$ .  
0.1235 gm. subst.; 16.35 cc. N at 21°, 768 mm. (nitrous acid method).

	Calculated for $C_9H_{11}O_2N$ :	Found:
C.....	59.67 per cent	59.73 per cent
H.....	6.08 per cent	6.21 per cent
N.....	7.73 per cent	7.58 per cent

By further concentration a second crop of 0.053 gm. was obtained making the total yield 1.078 gms., or 3.48 per cent.

*Analysis:* 0.0530 gm. substance; 0.1151 gm.  $CO_2$ ; 0.0300 gm.  $H_2O$ .  
C, 59.23 per cent; H, 6.29 per cent.

### *Hexone Bases by the Osborne Modification of Kossel's Method*

18.80 gms. of albumose were hydrolyzed; and the hexone base precipitated by phosphotungstic acid at 2 liters dilution in presence of 5 per cent sulphuric acid. The precipitate was freed from phosphotungstate and sulphate by means of barium chloride, and the histidin, arginin, and lysin determined by the Kossel-Patton method as modified by Osborne, Leavenworth and Brautlecht.<sup>1</sup>

The histidin solution was brought to 25 cc.; determinations were made of total nitrogen by the Kjeldahl method, and of primary amino nitrogen by the nitrous acid method. As has been shown in pure histidin the ratio, total nitrogen: amino nitrogen, is 3 and consequently small amounts of histidin in pure solution can be analyzed by determining this ratio. The following results were obtained on the histidin solution:

Amino N: 10 cc. solution; 11.85 cc. N at 29°, 758 mm. Amino N in total cc. is 0.01605 gm.

Total N: 15 cc. solution; 20.35 cc.  $\frac{N}{100}$   $H_2SO_4$ . Total N in 25 cc. solution 0.0476 gm.

Total N: Amino N = 2.97:1

The histidin solution was evidently pure. The total nitrogen corresponds to 0.176 gm. histidin, or 0.93 per cent.

The arginin solution was brought to 250 cc. volume. The ratio, total N: amino N, is 4:1 in the case of arginin. Determinations

<sup>1</sup> *Amer. Journ. of Physiol.*, xxiii, p. 180, 1908.

<sup>2</sup> Van Slyke: *Proc. Soc. Exp. Biol. and Med.*, Dec. 15, 1909.

tion of amino and Kjeldahl nitrogens serves here to check the purity of the arginin solution. Also, it has been found that one-half of the arginin nitrogen is quantitatively evolved in the form of ammonia during 6 hours boiling with 25 per cent NaOH under reflux. The greater part of the ammonia diffuses into standard  $\text{H}_2\text{SO}_4$  in a Folin 3-bulb tube at the top of the condenser, the remainder being distilled off later after addition of water to the alkaline solution. The following determinations were made on the arginin solution:

Total N: 20 cc. solution; 23.50 cc.  $\frac{\text{N}}{100} \text{H}_2\text{SO}_4$ . Total N = 0.4010 gm.

Amino N: 10 cc. solution; 9.70 cc. N at  $25^\circ$ , 764 mm. Amino N = 0.1353 gm.

Arginin N<sup>1</sup>; 40 cc. solution; 19.05 cc.  $\frac{\text{N}}{100} \text{H}_2\text{SO}_4$ . Arginin N = 0.3330 gm.

$\text{NH}_3$ ; 40 cc. solution; boiled with Mg O; 0.00 cc.  $\frac{\text{N}}{100} \text{H}_2\text{SO}_4$ .  $\text{NH}_3$  absent.

The ratio, total N: amino N, is lower than 4:1, and the arginin determination indicates that only 83.1 per cent of the total nitrogen present was in the form of arginin. Calculated on this basis, the arginin present was 1.036 gms., or 5.50 per cent.

The solution not used in the above determinations was concentrated and treated with an equivalent of picrolonic acid in alcoholic solution. 0.9 gm. of arginin picrolonate were obtained.

Analysis: 0.1440 gm. subst.; 0.2297 gm.  $\text{CO}_2$ ; 0.0696 gm.  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{10}\text{H}_{23}\text{N}_5\text{O}_7$ :	Found:
C.....	43.82 per cent	5.05 per cent
H.....	43.50 per cent	5.41 per cent

From the lysin solution 2.44 gm. of lysin picrate were obtained, equivalent to 0.95 gm. of lysin, or 5.06 per cent. The picrate was recrystallized from water, and gave the following figures on analysis.

0.1517 gm. subst.; 20.4 cc. N at  $18^\circ$ , 746 mm. (nitrous acid method).

	Calculated for $\text{C}_{12}\text{H}_{17}\text{N}_5\text{O}_9$ :	Found:
Amino N.....	7.47 per cent	7.58 per cent

<sup>1</sup> Van Slyke: *Proc. Soc. Exp. Biol. and Med.*, May 18, 1910. The decomposition of nearly one-half the arginin nitrogen into ammonia was noted by Osborne, Leavenworth and Brautlecht. The above method is quantitative.

*Determination of the Hexone Bases and Nitrogen Distribution by the Method of Van Slyke.<sup>1</sup>*

2.07 gms. of albumose containing 0.3413 gm. of nitrogen, were hydrolyzed with 20 per cent hydrochloric acid, the acid removed as completely as possible by evaporation, and the ammonia distilled *in vacuo* with an excess of barium hydrate solution. It neutralized 20.10 cc. of  $\frac{N}{10}$   $H_2SO_4$ .

The solution was acidified with the  $H_2SO_4$ , the melanin removed by adsorption with Ag Cl, and determined by Kjeldahl. 19.75 cc.  $\frac{N}{10}$   $H_2SO_4$  were neutralized.

The filtrate was brought to 100 cc. Portions of 5 cc. each were taken for determination of total and amino nitrogen.

*Total Nitrogen:* 10.10, 10.25 cc.  $\frac{N}{10}$   $H_2SO_4$ ; average 10.18. Total N = 0.2856 gm.

*Amino Nitrogen:* 18.55, 18.70 cc. N at 21.5°, 764 mm. Amino N = 0.2116 gm.

To the remaining 80 cc. 4 cc. of concentrated sulphuric acid and 60 cc. of a 20 per cent solution of phosphotungstic acid in 5 per cent  $H_2SO_4$  were added. The mixture was made up to 200 cc. volume and heated until the precipitate was nearly dissolved, then allowed to stand three days. The phosphotungstates of arginin, histidin, lysin and cystin are precipitated under these conditions. The precipitate was washed with a solution containing 5 per cent  $H_2SO_4$  and 2 per cent phosphotungstic acid and decomposed with a slight excess of baryta water. The solution of bases was freed from Ba with  $CO_2$  and brought to a volume of 50 cc. 10 cc. were used for Kjeldahl and amino determinations, in each case the remaining 30 cc. being used, first to determine the arginin by alkaline decomposition as already described (p. 231), and then to determine the cystin sulphur by fusion with  $KNO_3$ . The results were:

*Phosphotungstate Precipitate.*

Total N; 10 cc. solution: 8.25 cc.  $\frac{N}{10}$   $H_2SO_4$ . Total N = 0.0722 gm. = 21.15 per cent. of the total N.

Amino N: 10 cc. solution, 11.40 cc. N at 24°, 754 mm. Amino N = 0.0394 gm.

Arginin: 30 cc. solution; 6.80 cc.  $\frac{N}{10}$   $H_2SO_4$ . Arginin N = 0.0397 gm. = 11.62 per cent. of the total N.

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<sup>1</sup> Preliminary description, *Proc. Soc. Exp. Biol. and Med.*, May 18, 1910. The method will shortly be published in greater detail in this *Journal*.



The non-amino nitrogen ( $\frac{2}{3}$  of arginin N +  $\frac{1}{3}$  of histidin N) is  $0.0722 - 0.0394 = 0.0328$  gm. Subtracting three-fourths of the arginin N from the non-amino N, gives 0.0031 gm. as the non-amino histidin N, or 0.0046 gm. as the total histidin N, 1.34 per cent of the total nitrogen.

The difference (lysin and cystin N) between the total "basic" N and the histidin N + arginin N is 0.0279 gm. The 30 cc. of solution used for arginin and cystin determinations gave, for the latter 0.0790 gm. BaSO<sub>4</sub>, indicating 0.0099 gm., 2.90 per cent, of cystin N. This leaves 0.0180 gm., or 5.28 per cent, for the lysin N.

By subtracting the nitrogen in the phosphotungstic precipitate from that found in the solution before precipitation, the nitrogen of the "mono-amino acid" fraction is obtained. This method avoids the difficulties of Kjeldahling solutions containing phosphotungstic acid. The total N of this fraction is 0.2134 gm. = 62.54 per cent. Of this, 0.1722 gm., or 50.48 per cent, is amino nitrogen, 0.0412 gm., or 12.06 per cent, is non-amino nitrogen, consisting of the nitrogen of prolin, oxyprolin, one-half the tryptophan and perhaps some yet unknown acid or acids.

The results are summarized as follows:

	GRAMS NITROGEN	PER CENT OF TOTAL NITROGEN
Ammonia.....	0.0281	8.23
Melanin.....	0.0276	8.08
Arginin.....	0.0397	11.62
Histidin.....	0.0046	1.35
Lysin.....	0.0180	5.28
Cystin.....	0.0099	2.90
Amino N in phosphotungstic filtrate....	0.1722	50.48
Non-amino N in phosphotungstic filtrate	0.0412	12.06

The hexone base determinations, calculated for percentages of amino-acids in the dry albumose, compare as follows with those obtained by the older method.

	KOSSEL-PATTON- OSBORNE METHOD	NEW METHOD	MEAN
Arginin.....	5.50	5.96	5.73
Histidin.....	0.95	0.82	0.89
Lysin.....	5.06*	4.54	4.80

\* Lysin was calculated from unrecrystallized picrate, so the result may be slightly high.

Correction for the solubility of the phosphotungstates, which practically the same in both methods, as the concentration of precipitation were alike, increases the average arginin to 6.3 per cent, the histidin to 1.76 per cent.

The results of the hydrolysis are summarized in the following table, expressed in grams of amino-acid from 100 gms. of albumin.

Glutaminic acid.....	9.51	Aspartic acid.....	
Leucin.....	3.05	Glycocoll.....	
Isoleucin.....	2.96	Tyrosin.....	
Valin.....	3.54	Arginin.....	
Alanin.....	3.39	Histidin.....	
Valin-Alanin Mixture.....	1.86	Lysin.....	
Prolin.....	4.27	Cystin.....	
Phenylalanin.....	2.45	Ammonia.....	
		Total.....	

## NOTE ON INSOLUBLE LEAD SALTS OF AMINO-ACIDS.

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New York.)

(Received for publication, August 15, 1910.)

During preliminary studies on the lead salts of the amino-acids it was noted that tyrosin and aspartic acid form nearly insoluble compounds with lead when subjected to thorough boiling with precipitated oxide for 15–20 minutes. 0.2 gm. of tyrosin was boiled with 100 cc. of water and an excess of precipitated lead oxide. The nitrogen in the filtrate, determined by the Kjeldahl method, required, 2.13 cc.  $\frac{N}{10}$   $\text{H}_2\text{SO}_4$ , equivalent to 0.0376 gm. of tyrosin, about 1 part in 2600 of water.

It appeared that the precipitability of tyrosin by lead oxide might be used to separate tyrosin from the other amino-acids which may accompany it. A solution containing 1 gm. each of tyrosin, leucin and valin in 250 cc. of water was boiled with an excess of lead oxide. The precipitate was washed, then decomposed by boiling with water, to which sulphuric acid was added until an excess was present. The lead sulphate was filtered off, and the excess of sulphuric acid in solution removed by an exact equivalent barium hydrate. The solution was concentrated and 0.80 gm. tyrosin (7.68 per cent N) recovered.

Aspartic acid is similarly precipitated. The amount left in 100 cc. of solution after boiling 0.225 gm. of aspartic acid with an excess of lead oxide required only 1.55 cc.  $\frac{N}{10}$   $\text{H}_2\text{SO}_4$  in a Kjeldahl termination, indicating a solubility of about 1 part in 4700 of water.

Lead oxide has been frequently used to free mixtures of amino-acids from sulphuric and especially hydrochloric acids. From the

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above observations it appears that the process, if excess of  $\text{PbO}$  is used, is likely to remove aspartic acid and tyrosin from the solutions.<sup>1</sup>

<sup>1</sup> Abderhalden recommends boiling with  $\text{PbO}$  to remove  $\text{HCl}$  from the fraction of amino-acids, obtained by the ester method, which contains aspartic acid and serin (*Handbuch d. biochem. Arbeitsmethoden*, ii, p. 48).

# ON THE REFRACTIVE INDICES OF SOLUTIONS OF CERTAIN PROTEINS.

## II. THE PARANUCLEINS.

By T. BRAILSFORD ROBERTSON.

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(Received for publication, August 24, 1910.)

In previous communications<sup>1</sup> I have shown that the addition of relatively small amounts of casein, ovomucoid or ovovitellin to watery solutions of acids or bases very markedly increases their refractive indices; that this increase is directly proportional to the concentration of protein which is introduced into the solution and is independent of the acidity or alkalinity or temperature (between 20° and 40°) of the solvent, and that the change in the refractive index of a solvent which is brought about by the solution of one gram of one of these proteins in 100 cc. is characteristic for each of them. The values of this constant, which I designate by the symbol  $a$  are, for the above-mentioned proteins, as follows:

Casein.....	0.00152
Ovomucoid..	0.00160
Ovovitellin..	0.00130

I have extended these investigations to the series or mixture of bodies which results from the incomplete peptic hydrolysis of casein, and to which the collective term *paranuclein* has been applied,<sup>2</sup> and also to the bodies which result from the action of high concentrations of pepsin upon the products of the complete peptic hydrolysis of casein. The following are the results:

<sup>1</sup>T. Brailsford Robertson: *Journ. of Physical Chem.*, xiii, p. 469, 1909; *Journal. of Indust. and Engineering Chem.*, No. 10, Oct., 1909; this *Journal*, viii, p. 359, 1910.

<sup>2</sup>Cf. the literature cited by Gustav Mann: *Chemistry of the Proteids*, 1906, pp. 395-396.

## I. PARANUCLEIN.

To 8000 cc. of  $\frac{N}{10}$  potassium hydrate which had been rendered just acid to rosolic acid by the addition of casein (about 40 gms. per liter) were added 4 gms. of Gruebler's pepsin puriss. sicc. which had previously been dissolved in a little distilled water. After the addition of excess of toluol and thorough agitation the mixture was kept at 36° for 6 days. At the end of that time a thick white precipitate had formed and remained suspended within the fluid. The mixture was then heated to 100° in a steam-sterilizer for one half hour, cooled and allowed to stand at 36° for 24 hours. At the end of that time a thick white precipitate had settled to the bottom of the container. This was filtered off, washed in a large volume of distilled water and set aside under alcohol for 5 months. To this was then added the similar precipitate which was obtained by treating 6 liters of  $\frac{N}{10}$  potassium hydrate containing about 4 per cent of casein with pepsin for a week (adding 2 gms. every two days) and similarly prepared. After a few days the supernatant alcohol was poured off from the combined precipitates. They were then suspended in 8 liters of distilled water and sufficient sodium hydrate was added to render the solution tenth-normal. The entire precipitate dissolved readily, forming a clear yellowish solution. To this, after rapid filtration through glass wool, were added 60 cc. of glacial acetic acid. The thick white precipitate which was obtained was washed with 10 liters of distilled water, agitated thoroughly and allowed to settle in tall glass cylinders. The supernatant water was then removed by decantation and this process was repeated six times. The precipitate was then washed in 8 liters of Kahlbaum's 99.8 per cent alcohol in two successive washings. It was then collected on a hardened filter-paper and washed with about 4 kilos of Kahlbaum's ether (ueber Natrium destilliert). Finally, it was placed in a mortar and triturated with 1 kilo of ether; the supernatant ether was poured off and the precipitate was dried at 30° over calcium chloride and then at room-temperatures over sulphuric acid. The paranuclein was thus obtained in the form of a fine, light, friable, white powder.

Five grams of this substance were dissolved in 250 cc. of  $\frac{N}{10}$  potassium hydrate and this solution was diluted with  $\frac{N}{10}$  potassium hydroxide to the concentrations desired. The refractive indices

of these solutions were then measured at 22° in a Pulfrich refractometer, reading the angle of total reflection to within 1'. A sodium flame was employed as the source of light.

In the communications referred to above I have shown that for solutions of casein or of ovomucoid the refractive index is connected with the concentration of the protein by the formula:

$$n - n_1 = a \times c$$

$n$  being the refractive index of the solution of protein,  $n_1$  that of the solvent in which it is dissolved,  $c$  the percentage of protein in the solution, and  $a$  the constant referred to above, *i.e.*, the change in the refractive index of the solvent which is brought about by the addition of 1 gm. of protein to 100 cc. From the following table of results it will be seen that the same law holds good for solutions of paranuclein. The refractive index of  $\frac{N}{100}$  potassium hydrate at 22° is taken as 1.3334.

$c$ = CONCENTRATION OF PARANUCLEIN. PER CENT.	$n$ = REFRACTIVE INDEX OF SOLUTION.	$a = \frac{n - 1.3334}{c}$
2.00	1.3363	0.00145
1.50	1.3355	0.00140
1.00	1.3348	0.00140
0.75	1.3344	0.00133
0.50	1.3341	0.00140
	Average....	0.00140

It will be seen that the value of the ratio  $\frac{n - 1.3334}{c}$  is appreciably constant between 2 per cent and 0.5 per cent of paranuclein and equal to 0.00140.

## II. PARANUCLEIN A.

The phosphorus content of paranuclein is known to vary greatly with the circumstances under which it is prepared—the percentages of phosphorus which have been found varying from 0.88 to 6.86.<sup>1</sup>

<sup>1</sup> W. v. Moraczewski: *Zeitschr. f. physiol. Chem.*, xx, p. 28, 1895.

It appears probable, therefore, that the substance which has been termed paranuclein is, in reality, a mixture of two or more proteins resembling one another closely in their other properties but differing in their phosphorus content. I have found<sup>1</sup> that if paranuclein, prepared in the manner described above and containing 4.2 percent of  $P_2O_5$  be digested in excess of calcium hydrate, a substance of lower phosphorus content (1.6 per cent  $P_2O_5$ ) and somewhat less soluble in acids is produced. This substance I have provisionally termed Paranuclein A. If digestion with calcium hydrate be continued still further, or if it be carried out at a higher temperature, all substances precipitable by acetic acid disappear from the solution (*e. g.*, digestion for 18 hours at  $34^\circ$  of a 0.25 per cent solution of paranuclein in saturated calcium hydrate). It therefore appears possible that in successive hydrolytic cleavages the paranuclein which is first produced in a peptic digest successively loses phosphorus, which is split off as phosphoric acid,<sup>2</sup> until, finally, a substance or substances are produced which are no longer insoluble in neutral or faintly acid solutions, *i. e.*, which have lost one of the characteristic properties of the paranuclein group.

Paranuclein A was prepared in the following manner: Ten grams of paranuclein were dissolved in 4 liters of saturated calcium hydrate and the mixture, in the presence of excess of toluol, was allowed to stand at room-temperature for 16 hours. Seventeen cc. of glacial acetic acid were added and an abundant flocculant precipitate resulted, part of which settled to the bottom of the container, the remainder floating to the top. On agitating the mixture and allowing it to settle again the whole of the precipitate sank to the bottom. This was washed several times by decantation with water, and then with alcohol (99.8 per cent, Kahlbaum). It was observed that the precipitate shrank in volume upon the addition of the alcohol and that from being white it became brownish—the precipitate also tended to float on the top of the alcohol. The precipitate was collected on a hardened filter-paper, washed with 5 liters of 99.8 per cent alcohol and 2 liters of ether (Kahlbaum's *ueber Natrium destilliert*) and dried at  $30^\circ$  over calcium chloride and then at room-temperatures over sulphuric acid. Paranuclein

<sup>1</sup> T. Brailsford Robertson: this *Journal*, iii, p. 95, 1907.

<sup>2</sup> Salkowski and Hahn: *Arch. f. d. ges. Physiol.*, lix, p. 225, 1895.



A was thus obtained in the form of light grayish-brown powder or cakes which were easily pulverized. The estimated quantity of the product was 4 gms.

One gram of this substance was dissolved in 100 cc. of  $\frac{N}{50}$  potassium hydrate and 50 cc. of this were diluted to 100 cc. with  $\frac{N}{50}$  potassium hydrate. The refractive indices and values of  $a$  for these solutions were identical with those of the 1 per cent and 0.5 per cent solutions of paranuclein, namely:

$c$ = CONCENTRATION OF PARANUCLEIN A.	$n$ = REFRACTIVE INDEX OF SOLUTION.	$a = \frac{n - 1.3334}{c}$
1.00	1.3348	0.00140
0.50	1.3341	0.00140

It is therefore impossible to distinguish between paranuclein and Paranuclein A by the change which their presence causes in the refractive index of an alkaline solution.

### III. THE SUBSTANCE WHICH IS SYNTHESISED THROUGH THE ACTION OF PEPSIN AT 36° UPON THE CONCENTRATED PRODUCTS OF THE COMPLETE PEPTIC HYDROLYSIS OF CASEIN.

I have shown<sup>1</sup> that if the filtered products of the complete peptic hydrolysis of an approximately 4 per cent solution of casein in  $\frac{N}{50}$  sodium or potassium hydroxide be concentrated five or six times and 30 cc. of a 10 per cent solution of Gruebler's pepsin puriss. sicc. be added to 70 cc. of the concentrated solution thus obtained, after a lapse of from 2 to 48 hours a white precipitate is formed within the mixture, which when collected and purified, is found to very closely resemble Paranuclein A in its properties and phosphorus content. In the publications alluded to above I have brought forward evidence tending to show that in this case we have a real synthesis of protein from the products of its hydrolytic cleavage, and I have endeavored to interpret the mechanism which accomplishes this synthesis.

<sup>1</sup> T. Brailsford Robertson: this *Journal*, iii, p. 95, 1907; v, p. 493, 1909.

The reason why Paranuclein A is produced rather than casein or a paranuclein of higher phosphorus content is probably somewhat as follows. In event of synthesis occurring in a mixture such as that described above, the first substance which resulted which was insoluble in faintly acid solutions, would necessarily be thrown out of the sphere of action and the reaction would terminate at this point. Hence, from what has been said above, it is evident that were a member of the paranuclein group produced in these mixtures it would be Paranuclein A, rather than one of the other members of the group of higher phosphorus content.

It appeared of interest to ascertain the value of  $\alpha$  for this synthetic substance, with a view to comparing it with the value of the same constant for Paranuclein A.

Forty-two hundred cc. of the filtered products of the complete peptic hydrolysis of a 4 per cent solution of casein in  $\frac{N}{10}$  sodium hydrate free from substances precipitable by acetic acid either with or without the previous addition of alkali, were evaporated to 600 cc. To 340 cc. of this solution were added 135 cc. of 10 per cent pepsin (Gruebler's puriss. sicc.) and the mixture was set aside at 36° in the presence of excess of toluol. Within 15 hours a precipitate had appeared in the fluid. After 48 hours the precipitate was collected on a filter, and washed with water until the washings were colorless. About 200 cc. of water containing 30 cc. of  $\frac{N}{10}$  sodium hydrate were then poured into the filter and the contents agitated while the drippings were caught in about an equal volume of water containing 75 cc.  $\frac{N}{10}$  acetic acid. The resultant precipitate was collected on a filter, washed with water, and redissolved and precipitated as above. This precipitate was then washed with 1 liter of water, 2 liters of 99.8 per cent alcohol and 1 liter of ether (u.N.d.) and dried at 36° over calcium chloride and then at room-temperatures, over sulphuric acid. The substance thus obtained was a friable white powder very faintly tinged with yellow.

One gram of this substance was dissolved in  $\frac{N}{10}$  potassium hydrate. The refractive index of this solution was identical with that of a 1 per cent solution of paranuclein or of Paranuclein A, namely 1.3348, and for this substance also, therefore, the value of  $\alpha$  is 0.00140.

IV. THE SUBSTANCE WHICH IS SYNTHESISED THROUGH THE ACTION OF PEPSIN AT 60° UPON THE UNCONCENTRATED PRODUCTS OF THE COMPLETE PEPTIC HYDROLYSIS OF CASEIN.

In a previous communication I have shown<sup>1</sup> that it is possible to synthesise a substance apparently identical with the above and closely resembling Paranuclein A from the *unconcentrated* solution of the products of the complete peptic hydrolysis of 4 per cent casein in  $\frac{N}{10}$  sodium or potassium hydroxide, provided the synthesis be carried out at a high temperature (60° to 70°) and in the presence of considerable excess of pepsin. These temperatures are from 10° to 15° in excess of the temperature at which, according to the majority of observers, pepsin is rapidly and completely deprived of its proteolytic activity.<sup>2</sup> From this and from other evidence pointing in the same direction I have argued that the synthesis which occurs in these solutions is not an example of true "reversion" of a catalysed reaction, but that it is due to a shift in the equilibrium between the protein and its products consequent upon a shift in the equilibrium between two forms of the enzyme, one of which accelerates the hydrolysis and the other the synthesis of the protein. It appeared of interest to ascertain the value of  $\alpha$  for this substance also.

To 1500 cc. of the filtered and unconcentrated products of the complete peptic hydrolysis of 4 per cent casein dissolved in  $\frac{N}{10}$  sodium hydrate, free from substances precipitable by acetic acid, were added 300 cc. of 15 per cent pepsin (Gruebler's puriss. sicc.) and excess of toluol, both solutions having previously been heated to 60°. Within three hours a heavy precipitate had appeared within the mixture. After 48 hours the precipitate was collected on a filter and thereafter this substance was prepared in exactly the same manner as the substance described above. The product which is thus obtained is a light greyish-white, friable powder.

One gram of this substance was dissolved in  $\frac{N}{10}$  potassium hydrate. The refractive index of this solution was identical with

<sup>1</sup> T. Brailsford Robertson: this *Journal*, v, p. 493, 1909.

<sup>2</sup> Cf. Oppenheimer: *Ferments and their Actions*, trans. by Ainsworth Mitchell, London, p. 92, 1901; A. E. Taylor: On Fermentation, *Univ. of Calif. Publ., Pathol.*, i, p. 252, 1907; Schwarz: *Beitr. z. chem. Physiol. u. Pathol.*, vi, p. 524, 1905.

that of a 1 per cent solution of paranuclein or of Paranuclein A, namely, 1.3348, and for this substance also, therefore, the value of  $a$  is 0.00140.

#### CONCLUSIONS.

(1) The refractive indices of solutions of paranuclein, prepared from casein in the manner described in the body of the paper, are connected with their concentrations by the formula:

$$n - n_1 = a \times c$$

where  $n$  is the refractive index of the solution,  $n_1$  is the refractive index of the solvent, in this instance  $\frac{N}{D}$  potassium hydroxide (1.3334 at 22°),  $c$  is the percentage concentration of the protein in the solution, and  $a$  is a constant which is numerically equal to the change in the refractive index of the solvent which is brought about by dissolving 1 gm. in 100 cc. The same law has previously been shown to hold good for solutions of casein and of ovomucoid in various solvents.

(2) The value of  $a$ , in the above formula, for paranuclein is 0.00140.

(3) "Paranuclein A" is prepared from paranuclein by partial digestion with calcium hydrate and differs from it mainly in its lower phosphorus content. It is impossible to distinguish between paranuclein and Paranuclein A by the change which their presence causes in the refractive index of an alkaline solution, since for this substance the value of  $a$  is also 0.00140.

(4) I have previously shown that a substance is synthesized through the action of pepsin at 36° upon the concentrated products of the complete peptic hydrolysis of casein which closely resembles Paranuclein A in its properties. It is shown in this paper that this substance also resembles Paranuclein A in its effect upon the refractive index of an alkaline solution, the value of  $a$  for this substance being also 0.00140.

(5) I have previously shown that a substance is synthesized through the action of pepsin at 60° upon the *unconcentrated* products of the complete peptic hydrolysis of casein, which is a

parently identical with the above-mentioned substance and with Paranuclein A. In this paper it is shown that for this substance also the value of  $\alpha$  is 0.00140.

(6) These data may be regarded as affording confirmation of the view that the above-mentioned substances, synthesised through the action of pepsin from the products of the complete peptic hydrolysis of casein, are members of the paranuclein group.



# ON THE METABOLISM EXPERIMENT AS A STATISTICAL PROBLEM.<sup>1</sup>

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*(From the Laboratory of Physiological Chemistry, Department of Animal Husbandry, University of Illinois. Nutrition Investigations Publication No. 28.)*

(Received for publication, July 30, 1910.)

It is a generally recognized fact that two men to all appearances under identical conditions and of similar size, physique, constitution, temperament, etc., will often, on experimental investigation, yield considerably discordant results as to utilization of the food, composition of the feces and urine, nitrogen, phosphorus and sulphur retention, water elimination, changes in body weight and, perhaps more especially, structural composition of the blood, resistance to toxemia or bacteremia, intestinal bacterial flora, composition of the digestive juices, rate and character of digestion, etc. Again, it is well known that the same individual organism, under apparently identical conditions, will not behave in an identical manner at different times, variation being more or less pronounced depending upon the phase of metabolism considered. Such physiological behavior, peculiar to each individual organism and neither amenable to prediction or control, is ordinarily collectively designated as "individual idiosyncrasy." This property of living organisms of responding differently within more or less wide limits to identical conditions in so far as we can control conditions, we shall designate by the expression "functional variability."

The numerous experiments that have been performed to deter-

<sup>1</sup> To avoid possible misunderstanding, it may be said that by "statistical problem" is merely meant a problem that depends for its solution upon the accumulation of a large amount of data, this circumstance being the result of certain characteristics inherent in the phenomena with which the problem is concerned.

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mine the minimum level of protein metabolism at which a nitrogen balance may be retained, afford a good illustration of functional variability and the extent to which it may vitiate general conclusions. The remarkable contrast existing between the results obtained by Klemperer, Siv  n, Peschel, Caspari and Glaessner, Hirschfeld and Chittenden, on the one hand, and those of Neumann, Breisacher, Caspari, Ritter and Kumagawa, on the other, can be largely explained on the assumption that individual organisms have their own peculiar minimum requirements and exhibit varying degrees of inertia on being reduced to such minima.

In any investigation to determine the effect of drugs, preservatives, dietary modifications, etc., upon health or metabolism experience has shown that considerable allowance must be made for the uncertainties of functional variability. In an experiment that was undertaken by this laboratory in 1907-08, the results of the analysis of the urinary excretion of six men under largely uniform conditions for a period of 96 days, indicate the following variabilities.

TABLE I.  
COEFFICIENTS OF VARIABILITY.

(a) *of the Average Quantities of Various Forms of Urinary Nitrogen Excreted in 24 Hours.*

FORMS OF NITROGEN	SUBJECT 1	SUBJECT 2	SUBJECT 3	SUBJECT 4	SUBJECT 5	SUBJECT 6
Urea nitrogen.....	6.9	8.2	7.7	8.2	7.0	6.8
Ammonia nitrogen.....	6.7	13.5	9.4	7.9	11.5	7.9
Creatinin nitrogen.....	6.9	4.3	5.7	6.6	7.7	5.6
Uric acid nitrogen.....	4.2	4.8	11.5	5.8	8.4	7.4

(b) *of the Average Percentages of the Total Urinary Nitrogen Contained in Various Urinary Constituents as Excreted per 24 Hours.*

URINARY CONSTITUENTS	SUBJECT 1	SUBJECT 2	SUBJECT 3	SUBJECT 4	SUBJECT 5	SUBJECT 6
Urea.....	3.6	3.7	3.5	4.2	3.2	3.3
Ammonia.....	7.5	13.3	10.8	10.3	14.8	8.1
Creatinin.....	4.3	8.8	10.9	8.7	8.3	8.1
Uric Acid.....	6.5	9.0	12.7	7.6	8.0	9.4

NOTE: The coefficient of variability is the standard deviation in per cent of the mean; standard deviation being the square root of the average of the squares of deviations from mean of the separate observations.



This period extended from March 24 to June 28. The menu was practically constant and the same for all of the men and the food intake of each varied but slightly. The urine was analyzed in 4-day composite samples. It is, therefore, reasonable to conclude that the coefficients obtained are largely an expression of functional variability.

Perhaps the most striking illustrations of the subject under discussion are offered by those experiments upon the physiological resistance of the animal organism to the effects of hemorrhage, infection, poisoning, under-feeding or inanition, etc., as well as the rapidity and ease of recuperation from such treatment. In this connection we may cite the experiments of Müller<sup>1</sup> upon the effect of inanition and variously modified diets upon the agglutinin content of the blood and its production of anti-bodies after inoculation with different species of bacteria, his subjects being pigeons. In no single experiment was a unanimous verdict obtained from the 10 to 17 pairs of pigeons employed, while, in the majority of cases, the results were far from this ideal. Recently, Foster has published the results of an extensive experiment on dogs to determine the influence of different proportions of protein in the food on resistance to the toxicity of ricin and on recuperation from hemorrhage.<sup>2</sup> In the words of the observer himself it appeared that, "within relatively wide limits the total amount of food, as well as its total content of protein, were less important factors in determining the animal's resistance in these particular experiments than the peculiar cellular processes which, for lack of a better and more definite term, may be called 'individual idiosyncrasy.'"

It is this property of functional variability as defined above, inherent in living matter, that renders the problems of metabolism statistical, and in any metabolism experiment this property undoubtedly has been an obstacle, oftentimes a serious obstacle, to the formulation of legitimate conclusions.

Let us consider, for example, an experiment to determine the effect of muscular exertion upon the excretion of some urinary constituent. A group of men is selected and investigated as to that urinary constituent, for a certain period of time under normal and

<sup>1</sup> *Arch. f. Hyg.*, li, p. 365. *Centralbl. f. Bakt.*, 1903.

<sup>2</sup> *This Journal*, vii, p. 379, 1910.

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definitely known conditions. A second series of observations is then made under as nearly as possible identical conditions with the exception of a definite increase or decrease in physical activity. On comparing the two sets of data a difference is found in the average daily excretion of the substance under consideration. The question now arises, Is this difference in excretory functioning due to the difference in muscular activity that occurred, or is it merely an expression of that property of the organism of functioning differently in spite of no observable change in the aggregate of causes and conditions that determine the character and intensity of such activity?

If the difference between the two sets of observations is great as judged from previous experience, it is reasonable to conclude with a high degree of certainty that it was primarily due to the only apparently variable condition, *i.e.*, that of physical activity. If the difference is slight, however, our confidence in such a conclusion should be correspondingly diminished.

Assuming, however, that the former is true, and that we are well justified in concluding that, on the group of men examined, muscular exertion has produced an effect, the question then arises, Are we justified in extending this conclusion to men in general? If our group of men were of considerable size, normal in constitution and health, and chosen at perfect random, and if the behavior of all of the members were consistent, the extension of our first conclusion would be reasonably well founded. If such conditions were not fulfilled, however, as they seldom are in actual research: if our group of subjects were small, as is generally the case, or our individual results variable or even contradictory, as frequently occurs, we should hesitate to formulate a general law as to the effect of muscular exertion.

Such are the questions that must be answered in the interpretation of results of experimentation in metabolism, as well as other branches of physiology and experimental medicine. The necessity of rationally solving such problems cannot be over-emphasized. At the present time there seems to be a widespread satisfaction in leaving the matter entirely to the judgment of the investigator, aided to a greater or less degree by the hazy and uncertain recollection of his own past experience in research and the ill-defined ideas derived and accumulated from the experiments of others. It

thus come to pass that the idea of what constitutes normal functioning, or significant variation in normal functioning due to known changes in environment or treatment, or significant deviation from normal functioning that deserves the name of abnormal or pathological, is either entirely a matter of individual opinion or largely a matter of precedent. There can be no pretense that it is a matter of fact.

There is, undoubtedly, great need of a criterion to differentiate between significant differences and differences due to functional variability merely, and the lack of such a criterion is undoubtedly largely responsible for many of the controversies of the past and present; it undoubtedly is to blame for many of the contradictory conclusions of experimentation in metabolism, medicine and animal physiology. Its influence is especially to be traced in the investigations on the effects of drugs, preservatives, alcohol, intoxicating beverages, and dietary modifications on health and metabolism, for, in assigning a significance to any functional variations observed, an ideal opportunity is offered for the expression of any preconceived or biased opinions that are so liable to occur in approaching such problems. In the absence of any rational, systematic and generally accepted procedure in interpreting the results of such work, we find without exception that one or two tendencies may be traced in the attempt to decide whether a certain treatment is with or without effect, or beneficial or detrimental. First, the tendency may be to interpret the changes in metabolism observed, according to the quantitative values actually obtained, allowing nothing for the uncertainties of functional variability or even the limits of accuracy of analytical methods, and, on the other hand, the tendency may be to discount the actual changes that occurred by allowing for functional variability but by overestimating its extent, such estimations in all cases being, at best, mere guess work, tempered perhaps by a generous measure of scientific experience, but often, also, by an over-readiness to accept experimental results devoid of serious meaning.

This must not be understood as an attempt to impeach or even to question the conclusions of the large majority of the accepted conclusions of metabolism and the allied sciences, for they have, in great measure, been established beyond reasonable doubt by repeated verification. We are merely taking the stand that the current methods of deducing conclusions in these fields of research are open to considerable im-

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provement. In the following discussion, we hope to show that many of the problems that confront the physiologist, being statistical in their nature, are as amenable to mathematical treatment as the problems that confront the statistician in determining the status of a community and wherein its present condition differs from that of other communities or from that of itself at some previous time. In other words, we hope to show that the theory of probability is as applicable to the problems of the one as to those of the other.

It is beyond the scope of this paper to enter into a detailed discussion of the significance, applicability, or derivation of the exponential law of error, as it is generally designated. A brief summary, however, of the fundamental principles upon which it is based and the general nature of the theory of probability in its relation to biology, is almost necessary at this point in the discussion.<sup>1</sup>

The accumulation of statistical data in the past has revealed a striking property of natural phenomena which can be traced everywhere, to a greater or less extent, throughout the whole field of our experience. For example, births, marriages, crimes, suicides deaths, anthropometric or biometric measurements, meteorological changes—it scarcely seems to matter what phenomenon we observe, so long as we confine our attention to a few events or measurements we are unable to detect any regularity in occurrence or magnitude, but, when we consider the total for a long succession, a kind of order begins gradually to emerge and at last assumes a distinct and striking aspect. Wind and weather are seen to lose their proverbial irregularity when examined on a large scale. The length of a single life is familiarly uncertain, but the average duration of a number of lives is becoming, in an almost equal degree, familiarly certain.

It is also a matter of experience that many classes of things and processes, differing widely in their nature and origin, do nevertheless appear to conform with a considerable degree of accuracy to one and the same law. Let us fix our attention on one character of a population and take a set of observations, *e.g.*, on the stature

<sup>1</sup> A very good non-mathematical treatment of the theory of probability, with special reference to its relation to moral and social science, is to be found in John Venn's *Logic of Chance*, 1876. The reader may also refer with advantage to the following monograph prepared by one of us: *Statistical Methods*, by H. L. Rietz, appended to E. Davenport's *Principles of Breeding*.

These observations, if taken in large numbers, form a "frequency distribution" if grouped in classes covering equal divisions of the range of the observations, for example, equal divisions of length. With cartesian coördinates we plot the values of the character for the middle of each class as abscissas and the frequency of the class or its "frequency of occurrence" as ordinates; or, generally more convenient, represent the deviations of the middle of each class from the mean value as abscissas and the frequency as ordinates, and then pass as smooth a curve as possible through the points thus located. For many frequency distributions the points thus located are fitted approximately, at least, by a so-called normal probability curve.

The properties of the normal probability curve when the origin is placed at the mean, are the following. It cuts the  $Y$  axis at right angles, expressing the fact that, at the mean, there are a large number of values approximately equal; after a time it begins to decay rapidly towards the  $X$  axis, expressing the fact that the number of observations begin to grow less common as we recede from the mean; the  $X$  axis is an asymptote in both directions, expressing the fact that the magnitude, however remote from the mean, is strictly proportional to the square of the deviation; and, finally, the curve is symmetrical to the  $Y$  axis, expressing the fact that equal deviations from the mean, in excess or defect, tend to occur equally often in the long run. For different distributions the curve may assume different forms, the vertex being extremely obtuse or extremely acute, expressing a greater tendency to concentrate themselves about the mean, but on analysis, all such curves will be found to conform, with a considerable degree of accuracy, to the above general description. Laplace, Quêtelet, Herschel and other great mathematicians have derived the equation of this normal curve and all agree on the result, though they differ widely as to the hypotheses<sup>1</sup> upon which they base their derivations. The mathematical expression of the normal curve is ordinarily seen in the following form:

$$y = ke^{-h^2 x^2}$$

where  $x$  is any deviation from the mean,  $y$  the probability of occurrence,  $e$  the base of Napierian logarithms and  $h$  and  $k$

<sup>1</sup>For different hypotheses and derivations see Czüber: *Theorie der Messungsfehler*, pp. 99-111.

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constants to be determined from the statistical data. It may be reduced, however, to the following expression, which is practically much more convenient:

$$y = \frac{1}{\sigma \sqrt{2\pi}} e^{-\frac{x^2}{2\sigma^2}}$$

in which  $\sigma$  is the so-called "standard deviation" of the individual observations from the mean and is equal to

$$\sigma = \sqrt{\frac{\sum x^2}{n}}$$

$n$  being the total number of observations and  $\sum x^2$  the sum of the squares of the deviations from the mean value.

The above equation gives the probability of the occurrence of a deviation of any given magnitude from the mean, being expressed in decimals ranging from 0 to 1, the latter probability amounting to absolute certainty. Its meaning may be better appreciated by reference to the geometry of the curve. If the total area between the  $X$  axis and the curve is equal to unity, the probability that a deviation obtained from a random observation falls within the interval  $x$  to  $x + \delta x$  is equal to the rectangular area  $y\delta x$ ,  $y$  corresponding to some value of  $x$  within this interval.

From this equation a formula may be derived for calculating the so-called "probable error," "errors" in scientific dictionaries being extended to deviations of statistical variates from the mean value. If a series of measurements of any character that varies according to the normal law is obtained, the probable error of a single measurement may be defined as an interval symmetrically including the mean measurement, such that the probability is  $\frac{1}{2}$ , or, in terms of wagers,<sup>1</sup> the chances are even.

<sup>1</sup> A more definite idea of probability is conveyed when the latter is stated in the form of a wager than in the form of a ratio. The one is obtained from the other as follows: if the probability ratio is  $\frac{a}{a+b}$ , the wager is  $a$  to  $b$ ,

more conveniently,  $\frac{a}{b}$  to 1. In practical life, odds of several hundred to one that a certain event will happen or that a certain conclusion is justified, is, in most matters, considered as a certainty. Odds exceeding these, even to a large extent, can add but little to the confidence with which we regard a prediction.

any measurement taken at random would fall within it. The formula for such a probable error is the following:

$$E_s = 0.6745\sigma = 0.6745 \sqrt{\frac{\sum x^2}{n}}$$

The probable error of the mean measurement may be defined as an interval, symmetrically including the mean measurement, in which the chances are even that the mean of any equal random sample of measurements of that character would fall. Its formula is as follows:

$$E_m = \frac{E_s}{\sqrt{n}} = 0.6745 \sqrt{\frac{\sum x^2}{n^2}}$$

Referring again to the conception of probability as a fraction of total area between the normal frequency curve and the  $X$  axis, it follows from the above definitions that the probable error is related to an area bounded by the curve representing the frequency distribution of individual observations in the one case or means in the other, two ordinates on opposite sides and equidistant from the origin, and the  $X$  axis, such that the ratio of this area to the total area is equal to  $\frac{1}{2}$ . The probable error is equal to half the distance between the two ordinates.

The exponential equation affords us a means of determining fairly certain limits within which the probability is  $\frac{1}{2}$  that a deviation will fall, i.e., those set by the probable error, but also with any probability we may expect a deviation not to exceed any predetermined limit. Thus, taking limits that are multiples of the probable error, Gauss's Law of Error enables us to assert that, for observations that follow this law, the chances that another random observation or the mean of any equal random sample, will fall within the range  $\pm E$ ,  $\pm 2E$ , etc., are as follows:<sup>1</sup>

$\pm E$ the chances are	even.
$\pm 2E$ the chances are	4.5 to 1.
$\pm 3E$ the chances are	21 to 1.
$\pm 4E$ the chances are	142 to 1.
$\pm 5E$ the chances are	1310 to 1.
$\pm 6E$ the chances are	19200 to 1.
$\pm 7E$ the chances are	420000 to 1.
$\pm 8E$ the chances are	17000000 to 1.
$\pm 9E$ the chances are	about 1000000000 to 1.

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B. Davenport: *Statistical Methods*, p. 14.



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It is improbable, therefore, that the deviation of another random observation will exceed the probable error many times. In instance, it is practically certain that it will not exceed it  $n$  times, since the table shows that the chances are about a billion one against such a contingency; in other words, in the long run we would expect a deviation  $> \pm 9E$  only once in every billion trials *i.e.*, practically never.

Geometrically these wagers merely mean that the area symmetrically including the  $Y$  axis whose base is equal to  $2E$  is to the remaining area under the curve as 4.5 is to 1; that the area whose base  $3E$  is to the remaining area as 21:1; etc.

The normal law of error should be regarded as an ideal, which many classes of statistical data approximate to more or less closely. The justification for its general employment in evaluating statistical results is largely empirical. It is a mathematical expression of general experience, and, while it may not accurately represent the frequency distribution of any particular class of observations it certainly gives a valuable first approximation. In fact, as Morgan Crofton aptly says, "it is not to be supposed that the results arrived at when the calculus of probabilities is applied to most practical questions are anything more than approximations; but the same may be said of almost all such applications of abstract science. Partly from ignorance of the real state of the case, partly from the extreme intricacy of the calculations requisite if all the conditions which we do or might know are introduced, we are obliged to substitute in fact, for the actual problem, a simpler one approximately representing it."<sup>1</sup> We must not suppose, therefore, that any law of probability can lead to a "mathematically accurate" conclusion merely because the results of its application are conveniently pressed in mathematical symbols or because the "law" itself is expressed by means of an equation. It must be realized that the process of reducing the facts of experience to a mathematical form is a process of simplification, *i.e.*, a process of eliminating minor factors (minor factors, it is true, that would unduly complicate and render entirely impossible the discovery and expression of the governing law or the underlying system.

In the lack of sufficient data for an empirical justification of

<sup>1</sup> *Encycl. Brit.*, article on Probability.



applicability of the normal law to any particular phenomenon—and it may be said that a complete justification by this method is, in many cases, practically impossible, since it involves a vast amount of data—resort may be had to an examination of the nature and causes of deviations from the mean, with the idea of determining whether they are compatible with the assumptions upon which the exponential equation is based. The hypotheses employed by different mathematicians in deriving the equation are, as mentioned elsewhere, numerous. One set of such assumptions,<sup>1</sup> however, which has, perhaps, a more intelligible meaning for the biologist, is the following, namely, that deviations from the mean arise as a result of the combined action of a very large number of independent<sup>2</sup> sources of variation, of which each, if it operated alone, would produce variations of very limited consequence as compared with those that result from the combination of all the remaining sources.

It is evident from actual experiment that the number of component elements need not be very great in order that there should be some approximation to the normal law. In the opinion of Edgeworth, “the presumption that, wherever three or four independent causes coöperate, the law of error tends to be set up, is a legitimate hypothesis.” While such an *a priori* justification is in a sense unsatisfactory, when not supported by empirical evidence, it is certainly sufficient to establish the normal law as a reasonable working hypothesis.

Even to phenomena whose single variates do not follow Gauss's curve, the latter may be applied with sufficient accuracy for all practical purposes in obtaining the probable error of a mean, since means are likely to be normally distributed even if the original observations are not. Bearing on this point, Edgeworth says, “if numerous elements, each independently varying in one or more dimensions according to almost any law of frequency, are added together, the sum is apt to vary according to the normal law of

<sup>1</sup> Morgan Crofton: On the Proof of the Law of Error of Observations, London, 1870; *Phil. Trans.*, clx, p. 175.

<sup>2</sup> Two sources of variation are independent in the probability sense when the probability of their combined occurrence is equal to the product of the probabilities of their separate occurrences.

error,"<sup>1</sup> this being a statement of what is known as the law of large numbers.

From this brief discussion it is evident that the theory of probability is a most valuable supplement to the ignorance and frailty of the human mind in that "it seeks to determine the amount of reason which we may have to expect any event when we have not sufficient data to determine with certainty whether it will occur or not;"<sup>2</sup> any law of probability such as the normal law, is a mathematical expression for the prediction of the distribution of the varying results of future repetitions, though, like many an inference which we make with respect to a future event, it is more or less doubtful.

Having thus obtained a general idea of the significance and applicability of the law of error, in pursuance of the expressed object of this paper let us see if the theory of probability can reasonably be applied to observations of the functioning of living organisms. In ordinary life any metabolic function is largely determined by gross factors, generally few in number and profound in their influence. Thus, the character and quantity of digestive juices, the vigor of gastric and intestinal peristalsis, and the character and quantity of fecal and urinary excretion, depend to a considerable extent upon the character and quantity of the ingesta, both previous to and simultaneous with the functional act itself; the vigor and frequency of heart action varies markedly with the physical activity and mental condition; the heat production, the heat dissipation and the resultant body temperature are profoundly affected by physical exertion and external temperature and humidity, as are also the cellular oxidative processes as measured by the gaseous exchange. However, when all such gross factors are strictly controlled and kept at constant activity, when the food intake, physical and mental activity, and environment are as constant as can be, in fact, when all conditions, in so far as we are cognizant of and can control conditions, are eliminated as carefully as possible as factors in functional variation, functional variation still persists within more or less wide limits.

It is undoubtedly impossible, at the present state of our knowl-

<sup>1</sup> *Encycl. Brit.*, article on Law of Error.

<sup>2</sup> Mellor's *Higher Mathematics*, p. 490.

edge at least, to attempt a satisfactory and complete explanation of such residual variations which are an expression of what we have called functional variability, or to predict with certainty just to what extent one organism will duplicate its own functional behavior or that of another under apparently identical conditions. We must conclude that they are due to the sensitiveness of living matter to changes in environment that we are either not cognizant of or are unable to entirely control.

Among the numerous factors that cause such variation, many are due to conditions we cannot, or can only very crudely, control, it being either impossible or impracticable to secure perfect control. Mental, nervous and physical activity, as well as such characters of the environment as temperature, atmospheric pressure, humidity, light, sound, electro-magnetic condition, etc., are examples of this class of factors. Other influencing factors are due to our imperfect control of the grosser conditions. Even the food intake cannot be perfectly controlled, variations in composition, weight, temperature and volume being inevitable.

It seems a reasonable assumption, therefore, largely on account of the apparent haphazardness of these variations and their non-conformity to any general rule in so far as we know, that they are due to a large number, perhaps an infinite number, of unknown and conflicting causes which are just as liable in the aggregate, as far as we are able to predict, to act in one direction from the mean as in another: just as liable under apparently identical conditions to inhibit as to accelerate digestion, secretion, synthesis or disintegration of body tissue, or to decrease as to increase the acidity of the gastric juice, the leucocyte content of the blood, the utilization of the food protein, etc.

Upon this assumption, which is the logical resultant of our present knowledge, metabolic functioning falls legitimately within the province of probability. Further, the nature of the causes, or rather what we know of the causes, that determine such functioning, as discussed in the preceding paragraphs, conform remarkably well with certain hypotheses from which the normal law of probability may be derived, particularly with those of Morgan Crofton stated above.<sup>1</sup> We are therefore inclined to accept as a working

<sup>1</sup> See p. 307.

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hypothesis the assertion that the sum total of these numerous and variable components will follow the normal law of variation, i.e., Gauss's Law of Error. However, in view of Edgeworth's statement quoted on p. 307 and because of the fact that the reasonable evaluation of *average* observations is the principal aim of the application of any law of error to the data of metabolism, it is not necessary for the extension of Gauss's Law to the field of physiology that this working hypothesis be strictly satisfied. It is usual among statisticians when the data is not sufficient in amount to determine empirically the actual law of distribution of individuals and when no evidence argues against a normal distribution, to assume that the latter exists, because, in a very large number of cases, this gives an approximation so close that a small sample will give no real information as to the manner in which the population deviates from normality; since some law of distribution must be assumed it is better to work with a curve of general occurrence among biological phenomena whose area and ordinates are tabled and whose properties are well known.

In this circumstance we possess a means of minimizing and in many cases practically eliminating the obstacles above referred to in the interpretation of the results of experimentation in the field of metabolism. By calculating for a result of a series of observations (such as a mean) made under controlled conditions, its probable error, we provide ourselves with a measure of the significance of that result. We are then in a position to answer the question, If this series of observations be repeated under the same conditions, in so far as we can control conditions, how near to the first result ought we to expect a second to fall, or, perhaps more accurately, with what degree of confidence ought we to expect the second result to fall within any assigned limits from the first? Not knowing the answer to such a question, that is, not knowing the extent of functional variation due to uncontrolled conditions, the value of any result of a series of observations is doubtful.

Thus, in the example first cited,<sup>1</sup> the difficulties there referred to could be overcome by calculating the probable errors of the means of the two series of observations and from these the probable

<sup>1</sup> See p. 299.

error of the difference between the means<sup>1</sup>, a criterion being thus obtained of the real significance of this difference, *i.e.*, the form in which it might probably recur upon repetition of the experiment. For an experiment upon a group of subjects instead of an individual, there are two probable errors of the group mean, the two having totally distinct meanings. The one, the probable error with respect to time, obtained from the probable errors of the individual means by applying to them the formula for the probable error of an arithmetical mean, affords a prediction of the result of repeating the experiment with the same subjects under similarly controlled conditions and enables one to answer the question, in the hypothetical case instanced, Has muscular exertion affected the excretion *by this particular sample of men* of a certain urinary constituent "A"? The other, the probable error with respect to men, obtained by direct derivation from the individual means, affords a prediction of the result of repeating the experiment under similarly controlled conditions with a like group of other subjects selected at random from the same class of men, and enables one to answer the more general question, Does muscular exertion affect the excretion *by the human organism* of this substance "A"?

To anyone at all familiar with the actual conditions under which metabolism experiments have been and still are performed, it is evident that in but few exceptional cases do they correspond to the ideal conditions necessary for the strict applicability of the theory of probability.

Thus, in many experiments, most of the conditions are very loosely controlled, either necessarily or knowingly so, or both. Under such circumstances other causes than the accidental ones with which probability is properly concerned enter into the case, thus precluding the correct application of any law of error.

<sup>1</sup> The formulas for the propagation of error during various arithmetical processes, such as addition, subtraction, division, etc., may be found in Mellor's *Higher Mathematics for Students of Chemistry and Physics*, in Merriam's *Method of Least Squares*, and elsewhere. The main hypotheses upon which such formulas are based, *i.e.*, the assumption of large numbers and independence, are essentially those from which the normal law is derived, but are applied to the errors themselves or deviations from the mean rather than to the causes that determine such errors or deviations. It is almost needless to say that the more a probable error is removed from the original data by the employment of such formulas the less trustworthy it becomes.

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In the majority of experiments small periods of observation involving a meagre amount of data for any one phase of metabolism considered, are the general rule. While it is well known that the ordinary method of using the probable error and placing wagers as to the accuracy of results is only trustworthy when the random sample is fairly "large," it still remains rather indefinite as to where the distinction between "large" and "small" samples is to be drawn. The astronomer and the physicist do not, in general, hesitate to apply a probable error obtained from the assumption of a normal distribution to a dozen observations, while the statistician, in general, feels that he should deal with much larger numbers. Student<sup>1</sup> has recently made what seems to us an important contribution bearing on this point. In this paper he has dealt with the theory of small samples of 4 to 10, drawn from an indefinitely large population normally distributed. In certain cases where the experiments are too few to apply the usual formula for placing wagers on the significance of a mean, he furnishes an alternate formula and a table of probabilities for  $n = 4$  to 10, where  $n$  is the number in the sample drawn. His method gives the probability that the mean of the sample does not differ by more than  $z$  times the standard deviation of the sample from the mean of the population from which the sample is drawn,  $z$  being the ratio between the mean of the sample and its standard deviation. He further shows that, for the distribution of means of samples of larger size, the assumption of normality gives a curve of standard deviation  $\frac{s}{\sqrt{n-3}}$ , although it is rather common practice to accept the value  $\frac{s}{\sqrt{n}}$ , on the ground that the standard deviation of the means of samples of  $n$  is the standard deviation  $\sigma$  of the total population divided by  $\sqrt{n}$  and accepting  $s$ , the standard deviation of the sample, as an approximation to  $\sigma$ . For really large values of  $n$ , it matters little whether we use  $\frac{s}{\sqrt{n}}$  or  $\frac{s}{\sqrt{n-3}}$  in finding the probable error and in drawing a conclusion as to the significance of the results. For small values, however, the difference is of importance.

<sup>1</sup> *Biometrika*, vi, part I, pp. 1-25.

ent compares probabilities from his table for  $n = 10$  with results obtained from the normal equation of the distribution means of samples of  $n$  with  $n$  equal to 10 and the standard deviation equal to  $\frac{s}{\sqrt{n-3}}$ ,  $s$  being the standard deviation of the

and finds that the latter probabilities do not differ significantly from the former except for values of  $z > 0.8$ . For such values the normal curve gives a false feeling of security that the mean population is not significantly different from the mean of the sample. While Student has thus brought samples of small size almost completely within the sphere of the theory of probabilities and probable errors obtained from his tables his revised formula must generally be of such a size as to make a meagre sample of physiological data of little value because of the comparatively wide functional variability. As Student himself says, when speaking of the application of his method to the results of only two pot culture experiments and as thus obtained that a certain conclusion is justified, "these are those which would be laid, and laid rightly, by a man whose knowledge of the matter was contained in the two experiments. A conversant with pot culture would, however, know that the difference between the results would generally be greater and correspondingly moderate the certainty of his conclusion." In such cases, therefore, when the amount of data is obviously small from a common-sense standpoint, the method has no practical application, since the opinion to which it leads, no matter how logically deduced from the data at hand, is little better than a confession of ignorance.

In metabolism work of any kind small numbers of subjects are universally employed. This fact renders of little or no practical application of the probable error directly to the individual in order to predict what might be expected if the experiment were repeated upon a like group of other subjects, since a handful of men or dogs or guinea pigs cannot be regarded as approximately a fair sample of their respective species. The necessarily unfavorable conditions for sampling a population confront the physiologist as well as the statistician, must be fully realized at this point. They do not even approximate the conditions confronting the chemist. The latter can reduce his



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material, no matter how heterogeneous, to a practically homogeneous condition; then, upon withdrawing a small portion for direct examination, he may rest assured that within infinitesimal, oftentimes negligible, limits, such a portion, though small in size, is thoroughly representative of the whole.

A population is far from being amenable to such a procedure and yet it can only be conceived of as exhibiting marked heterogeneity, functional as well as morphological. The numerous influences, internal and external to the organism, that are constantly at work during the entire life of the individual, in fact during the life of his ancestry as well, now in one combination, now in another, can only result in the production of a distinct organism, unlike any other. Considering the development of the germ only, E. Davenport says, "it is not difficult to see many causes of variation in the internal processes known to be involved in the activities of the living protoplasm. Growth is the result of cell division, which seems to proceed upon plans calculated to insure qualitative as well as quantitative equality as between the daughter cells. Any deviation from the plan, however—and deviations are known to occur—must result in variation. This is especially true in the reduction process which is characteristic of maturation in both sexes."<sup>1</sup> Add to this the infinite hereditary potentialities contained in the sexual elements themselves, the variety of combinations possible during fertilization, the possible degrees of reversion of the resulting germ, and the environmental forces controlling, restricting and modifying its intra-uterine and later life, and we no longer marvel that variability is the most obvious fact about living beings.

While it is generally conceded that the morphology of a population presents a statistical problem, this does not seem to be so fully realized for its physiology. It is universally recognized that it is ridiculous to assert that the average stature of a random sample of 6, 12, or even 50 men is even approximately the average stature of the race, but if it is asserted as a fact by a person whose ability and honesty are beyond question that a certain substance, or a certain diet, or a certain treatment has had a certain physiological effect, beneficial, detrimental, or indifferent, upon a group of 6, 4.

<sup>1</sup> *The Principles of Breeding*, p. 216.



2 men, it is often regarded, even by the investigator himself, as a pretty definite proof that all men would be so affected; it takes but a slight amount of verification to place it in a variable position.

In either case, in either matter of fact there is no distinction between the two classes.

Their truly statistical character is a little more obvious in the former, perhaps, than in the latter, but why such a sharp distinction should be drawn is difficult to comprehend. We recognize functional variability in the terms "personal idiosyncrasy" or "personal equation," but somehow fail to appreciate its importance, extent, or its constant and universal tendency to vitiate inductive reasoning. The disposition to constantly generalize from individual experiences without duly considering their value and extent, seems to be a universal characteristic of human nature. And a few months, or weeks, or even days, in a foreign country, on return fully prepared to discourse upon the natural character of its inhabitants, the general condition of their civilization, and the improvements that might be made therein. Perhaps in the same way we obtain certain results from a metabolism experiment on a few men or dogs and are strongly inclined to announce to the world certain physiological facts concerning the human or dog organism.

In the face of variability, therefore, a population cannot be properly sampled unless a large number of individuals are taken, the size of the sample thus compensating for its lack of homogeneity. The statistician recognizes and it is this that the physiologist should recognize. It is undoubtedly a fact that Student's *t*-test may be applied to the data from a small number of individuals, regarding such as a sample of the population from which they are drawn, but, since the functional variability from subject to subject is generally greater than that from time to time, we must be sure that the probabilities or probable errors resulting from a sample of such size as to render such data of little value as compared with the whole from this standpoint. The observations above made in regard to the applicability of these methods to the data obtained from all experimental periods apply here with added force.

It is thus true that the theory of probability has but a limited application to experiments in metabolism as performed at the present day or, when applied, a limited value. This mere fact, how-

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ever, in connection with the general existence of functional variability, is a serious argument against the generally accepted value of short and incomplete investigations and the oftentimes sweeping conclusions deduced therefrom. As links in a developing chain of evidence such experiments undoubtedly possess great value, but as grounds for positive conclusions or general application they are seldom sufficient.

It is our firm belief that any argument against the applicability of the theory of probability to experimental data characterized as are the data of metabolism, is an argument against the value of the data themselves. If the data are meagre and yet variable, or if the conditions under which they were obtained were loosely controlled so that the grosser factors whose influences are not conjectural, are left in unhampered activity, it is a fact that the laws of probability cannot be enlisted, but it is just as truly a fact that the data themselves are devoid of significance. An experiment from which no rational conclusions can be drawn for the simple reason that we have no grounds for believing that similar or totally different results would be obtained upon repetition under similarly controlled conditions is certainly of dubious value.

The problems of metabolism are statistical in their nature, being concerned as they are with phenomena influenced by apparently accidental causes that produce, in quantitative measurements, deviations whose particular antecedents we are unable to give. Being thus characterized, they can seldom be solved in an unequivocal manner except after repeated corroboration or extensive investigation. While a single experiment, therefore, may not settle definitely the problem upon which it was launched, by applying the laws of probability, its conclusions may be stated in such a way as to point to *the most probable* solution at least. For this reason, the significance of investigations in this field can be determined to the fullest extent by the use of this method.

It is therefore important in planning such work to consider well the requirements for the strictest application of this theory. By obtaining a large number of observations upon any one phenomenon, by controlling carefully the grosser factors that determine its character and magnitude, and by eliminating as far as possible all systematic sources of variation except the one whose influence it is desired to determine, we will be in a position to deter-

mine a highly probable conclusion in relation to these subjects themselves, concerning the phenomenon investigated. By employing large groups of subjects or by repeating the experiment upon other subjects, we will be in a position to determine a highly probable conclusion in relation to the entire class from which the subjects were drawn. While it may be conceded that it is feasible to increase the duration of investigations and to increase the number of subjects or to verify the first by repetition upon others, it may be objected that it is impossible to sufficiently control conditions and thus eliminate the grosser disturbing factors. In answer to this possible objection, it may be said that only approximate elimination is necessary, since slight variations in these grosser factors due to incomplete control must themselves partake of the accidental and therefore fall within the legitimate province of probability.

In conclusion, we wish to illustrate by a practical problem the advantages of the probability method. Twelve men were placed for a period of 92 days from December 23 to March 23 under normal conditions. During the last 48 days of this fore-period, during which conditions were fairly constant, the creatinin was determined by Folin's colorimetric method in 4-day composite samples of urine. Starting March 24, the men were divided into two groups, the first, Group A, continuing under fore-period conditions, the second, Group B, also continuing under fore-period conditions with the exception of a definite dosage of saltpeter in the fresh meats served. This constituted the test period and continued for 96 days to June 28. With this brief introduction, we will proceed with the question, Has the ingestion of saltpeter by the members of Group B affected in any way their excretion of urinary creatinin.

The average creatinin-nitrogen excretion per 24 hours for the 12 men is given in Table 2. In Group A, with one exception, there was an increase in excretion, ranging from  $0.001 \pm 0.0079$  gm. to  $0.040 \pm 0.0084$  gm. or  $0.1 \pm 1.1$  per cent to  $6.3 \pm 1.3$  per cent. With the single exception of one member, however, the variations are but slightly if any greater than their probable errors and are, therefore, worthy of no significance, since we can feel no confidence that, upon repetition of the experiment, increases would be

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obtained.<sup>1</sup> We cannot assert, therefore, that, for the five men at least, the test period conditions were essentially different in regard to their effect upon the excretion of creatinin, from the fore-period conditions.

TABLE 2.  
AVERAGE AMOUNTS OF CREATININ NITROGEN EXCRETED PER MAN  
PER PERIOD.

*Results expressed in grams per 24 hours with their probable errors.*

GROUP A.

SUB- JECT	FEB. 5—MARCH 23	MARCH 24—JUNE 27	VARIATION IN GRAMS.	VARIATION IN PER CENT
1	0.648 ± 0.0041*	0.650 ± 0.0062	+ 0.002 ± 0.0074	+ 0.3 ± 1.1
2	0.637 ± 0.0072	0.677 ± 0.0043	+ 0.040 ± 0.0084	+ 6.3 ± 1.3
3	0.675 ± 0.0059	0.685 ± 0.0061	+ 0.010 ± 0.0085	+ 1.5 ± 1.3
4	0.686 ± 0.0066	0.668 ± 0.0056	− 0.017 ± 0.0086	− 2.5 ± 1.3
5	0.672 ± 0.0054	0.687 ± p.0077	+ 0.015 ± 0.0094	+ 2.2 ± 1.4
6	0.701 ± 0.0058	0.702 ± 0.0055	+ 0.001 ± 0.0079	+ 0.1 ± 1.1
Av...	0.670 ± 0.0024*	0.678 ± 0.0024	+ 0.008 ± 0.0034	+ 1.19 ± 0.51.

GROUP B.

SUB- JECT	FEB. 5—MARCH 23	MARCH 24—JUNE 27	VARIATION IN GRAMS.	VARIATION IN PER CENT
7	0.766 ± 0.0081	0.760 ± 0.0067	− 0.006 ± 0.0105	− 0.8 ± 1.4
8	0.655 ± 0.0059	0.664 ± 0.0070	+ 0.009 ± 0.0092	+ 1.4 ± 1.4
9	0.744 ± 0.0086	0.771 ± 0.0077	+ 0.027 ± 0.0115	+ 3.6 ± 1.5
10	0.654 ± 0.0069	0.671 ± 0.0076	+ 0.017 ± 0.0102	+ 2.6 ± 1.6
11	0.728 ± 0.0053	0.740 ± 0.0085	+ 0.012 ± 0.0100	+ 1.6 ± 1.4
12	0.626 ± 0.0028	0.608 ± 0.0058	− 0.018 ± 0.0081	− 2.9 ± 1.3
Av...	0.695 ± 0.0028*	0.702 ± 0.0029	+ 0.007 ± 0.0040	+ 1.01 ± 0.57

\*The probable errors of the group means are in respect to time (see p. 311).

<sup>1</sup> It is generally considered that a variation should exceed its probable error some four or five times at least before the accidental causes are allowed for. This merely means that, ordinarily, an event is only regarded as certain when it may be expected to occur some 1310 times out of 1311 trials or, at least, some 142 times out of 143 trials. While this is, indeed, largely a matter of opinion, the range within which opinion can operate is in this case extremely small, because of the fact that the wagers increase so rapidly (see p. 305).

In the case of Subject 2, the variation was  $+ 0.040 \pm 0.0084$  gm. or  $+ 6.3 \pm 1.3$  per cent, *i.e.*, from four to five times its probable error, indicating with a high degree of certainty that, for this individual, an essential difference existed between fore and test period conditions and that, if the experiment were repeated under similarly controlled conditions, a significant increase in creatinin excretion from fore to test period might be expected.

In Group B, with two exceptions, there was an increase in excretion from the first to the second period, though in all cases the probable errors of the period variations were of such magnitude as to render such variations devoid of significance.

In view of the fact, therefore, that in practically every case, for both groups alike, there was no significant change in creatinin excretion, one might well predict that the average variation would be insignificant. This was actually the case, the average for Group A being  $+ 0.008 \pm 0.0034$  gm. or  $+ 1.19 \pm 0.51$  per cent, and for Group B,  $+ 0.007 \pm 0.0040$  gm. or  $+ 1.01 \pm 0.57$  per cent. If significant average differences had resulted, however, a comparison between Groups A and B would have been necessary. The probable error employed in this experiment, however, could not strictly be applied to comparisons between two different groups of men, since they take no account of errors due to the random selection of the groups, *i.e.*, functional variability from man to man (see p. 311). On account of the smallness of the groups and the considerable variability that prevailed, a probable error that would allow for such variability is not feasible. Any comparison, therefore, that could be attempted between two such groups, one a test group and the other its control, would have to be done upon the assumption of a strict control relation such that if the test group had been treated in the same manner as the control group during the test period, its mean would have changed like the mean for the control group to within deviations determined by the probable error of such a change as calculated indirectly from the probable errors of the individual means, this probable error thus taking no account of the error in the random selection of the two groups. In any experiment in which a comparison is necessary between a test and a control group, both small in size, such an assumption of a strict control relation is always made, either tacitly or admittedly.

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It is quite possible that such an employment of this probable error might lead one to believe in some cases that significant differences existed between groups when, as a matter of fact, such differences may have been due merely to random sampling, in other words to variability from man to man. It is at least certain, however, that, if such a method renders group differences insignificant, we may feel convinced that they *are* insignificant.

In a comparison of the data of one group of men with those of another, there are objections to relying entirely upon group averages, especially if functional variability is very pronounced. For such a case, an individualistic method of comparison is highly desirable, such as the following:

This method concerns itself with the question, Is the deviation of the test group from its control group significantly greater or less in the test period than in the fore-period, due to the test treatment? Its details may be illustrated by its application to the creatinin data in the above table. On subtracting each of the individual fore-period averages of Group A from each of the individual fore-period averages of Group B, the 36 differences presented in the first section of Table 3 are obtained. These differences represent the deviation of the test group from its control group under like conditions and are merely an expression of the functional variability existing between the two groups due to random selection. If the test period individual averages of the two groups are subtracted in the same way, the differences presented in the second section of Table 3 are obtained. These differences represent the deviation of the two groups under test conditions and may be supposed to be due to the functional variability existing between them, plus a possible effect of the test treatment. The question that must now be answered is, Are the 36 test period differences significantly greater or less in the aggregate than the 36 fore-period differences?

Upon subtracting the fore from the test period differences, the values presented in the third section of Table 3 are obtained. These values represent the differences in period variation between the two groups. Their mean value is  $-0.00147$  gm. We have called attention to the fact that the large variations from subject to subject and the small number of subjects, make it practically useless to deal with the probable errors of group averages with respect to

TABLE 3.

COMPARISON BY DIFFERENCE OF THE DAILY EXCRETION OF CREATININ  
NITROGEN OF THE SUBJECTS OF GROUP B WITH THAT OF THE  
SUBJECTS OF GROUP A.

*Differences expressed in grams, + if the Group B weight is the greater, and -  
if the smaller.*

*Fore period. February 5 to March 23, 48 days.*

GROUP B	GROUP A					
	SUBJECT 1	SUBJECT 2	SUBJECT 3	SUBJECT 4	SUBJECT 5	SUBJECT 6
ect 7	+ 0.118	+ 0.129	+ 0.091	+ 0.080	+ 0.094	+ 0.065
ect 8	+ 0.005	+ 0.018	- 0.020	- 0.031	- 0.017	- 0.046
ect 9	+ 0.096	+ 0.107	+ 0.069	+ 0.058	+ 0.072	+ 0.043
ect 10	+ 0.006	+ 0.017	- 0.021	- 0.032	- 0.018	- 0.047
ect 11	+ 0.080	+ 0.091	+ 0.053	+ 0.042	+ 0.056	+ 0.027
ect 12	- 0.022	- 0.011	- 0.049	- 0.060	- 0.046	- 0.075

*Test Period. March 24 to June 28, 96 Days.*

GROUP B	GROUP A					
	SUBJECT 1	SUBJECT 2	SUBJECT 3	SUBJECT 4	SUBJECT 5	SUBJECT 6
ect 7	+ 0.110	+ 0.083	+ 0.075	+ 0.092	+ 0.073	+ 0.058
ect 8	+ 0.014	- 0.013	- 0.021	- 0.004	- 0.023	- 0.038
ect 9	+ 0.121	+ 0.094	+ 0.086	+ 0.103	+ 0.084	+ 0.069
ect 10	+ 0.021	- 0.006	- 0.014	+ 0.003	- 0.016	- 0.031
ect 11	+ 0.090	+ 0.063	+ 0.055	+ 0.072	+ 0.053	+ 0.038
ect 12	- 0.042	- 0.069	- 0.077	- 0.060	- 0.079	- 0.094

*Test Period minus Fore-Period.*

GROUP B	GROUP A					
	SUBJECT 1	SUBJECT 2	SUBJECT 3	SUBJECT 4	SUBJECT 5	SUBJECT 6
ect 7	- 0.008	- 0.046	- 0.016	+ 0.012	- 0.021	- 0.007
ect 8	+ 0.009	- 0.031	- 0.001	+ 0.027	- 0.006	+ 0.008
ect 9	+ 0.025	- 0.013	+ 0.017	+ 0.045	+ 0.012	+ 0.026
ect 10	+ 0.015	- 0.023	+ 0.007	+ 0.035	+ 0.002	+ 0.016
ect 11	+ 0.010	- 0.028	+ 0.002	+ 0.030	- 0.003	+ 0.011
ect 12	- 0.020	- 0.059	- 0.028	± 0.000	- 0.033	- 0.019



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variation from one subject to another, treating the means for individuals as a set of observations. The test period differences minus the corresponding fore-period differences leave only the changes in the differences between corresponding pairs of subjects. These changes for any pair would reduce to zero if this pair of subjects of A and B had maintained the same difference in the test period as they showed in the fore-period. If there is an influence on the function considered due to saltpeter, that applies to the group as a whole, it seems reasonable that the variations between these differences of the third section of the table may be small enough compared to their mean value to afford by the method of Student a prediction of some value in respect to the probable result of repeating this experiment on other subjects under similar conditions.

It should be noted that the above set of 36 differences are not independent, but can be obtained by linear combinations of 11 distinct elements of any row and column. These differences, however, are practically equally divided as regards sign, 18 being positive, 17 negative and 1 zero. Furthermore, in finding the mean  $-0.00147$  it is only necessary to select any one of the 720 groups of 6 differences that are formed by placing the 6 subjects of Group A in correspondence with all those of Group B.

In this case the differences are independent of each other, and we next inquire about the best value for the standard deviation of their distribution. If we should find the mean of the squares of the standard deviations of each of the 720 sets of 6, it can be proved algebraically that we should obtain the value of the standard deviation obtained from the 36 bound values in the third section of Table 3. This result is, we take it, a better value for the square of the standard deviation of the distribution of sets of 6 differences than could be expected from a single set of 6 differences.

Having thus the mean and standard deviation of our sample of differences we find the ratio  $z = \frac{0.00147}{0.023} = 0.064$ . We must

remember that the mean can be obtained from any 6 independent differences involving all the members of both groups, and it appears that we should therefore obtain the probability that the mean of the population of differences of which this is a sample, is negative,



by Student's table for  $n = 6$ .<sup>1</sup> For  $z = 0.1$  the wage rate mean of the population is negative is 58 to 42, and, for some value, the wage is still more nearly even, so that the effect of the population cannot be regarded as negative.

The procedure in finding the above wage is equivalent geometrically to determining the ratio of the larger area bounded by

now that we should enter this table for  $n = 6$ , let  $\bar{D}$  be the mean differences such as occur in the third section of Table 3. That is,

$\bar{D}$ , where  $D$  stands for any individual difference. Taking a new increment gives to each of these  $D$ 's an increment  $\delta D$ , and to  $\bar{D}$  an increment such that

$$\delta \bar{D} = \frac{1}{n} \sum_1^n \delta D \quad [1]$$

In the usual method of finding the standard deviation, we square the members, sum for a large number  $m$  of experiments, and divide by the number of experiments. This gives

$$\sum_1^m \frac{\delta \bar{D}^2}{m} = \frac{1}{n^2} \sum_1^n \sum_1^n \frac{\delta \bar{D}^2}{m} + \frac{2}{n^2} \sum_1^n \sum_1^n \delta D \cdot \delta D' \quad [2]$$

If the differences are independent deviations, we could assume these product terms to vanish to within limits due to random sampling. We cannot

do this for  $n = 36$ , for we cannot assume that product terms will vanish, on account of known correlation among the complete set of 36 differences. Even differences from which these 36 can be obtained by additions and subtractions, are not strictly independent in the probability sense. Moreover, they are obtained from any set of 6 independent differences involving all members of both groups. If we thus select differences, in the long run, the product terms in [2] vanish, and we obtain the usual expression for the probability of  $\bar{D}$  on the basis of  $n = 6$ .

$$\sigma_{\bar{D}}^2 = \frac{1}{n} \sum_1^n \frac{\sigma_D^2}{n} \quad [3]$$

As stated above, the average value for  $\sigma_D^2$  from all the sets of 6 is obtained as the standard deviation of the 36 differences of the third section of Table 3, and

$$\sigma_{\bar{D}} = \frac{1}{n} \bar{\sigma}_D$$

$\bar{\sigma}_D$  is the standard deviation of the 36 differences.

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the curve representing the frequency distribution of the means of samples of 6 drawn from a normal population (plotted with the origin at the mean value  $M$  of the sample), the ordinate  $X = -M$ , and the  $X$  axis, to the smaller area whose boundaries may be similarly described.

In regard to the general application of this method, it may be said that the table used gives values only for  $n = 4$  to 10. It is found that for  $n = 10$  and  $z > 0.8$ , the value is not materially different from what we would obtain on the assumption that the distribution of samples is normal, provided we divide the standard deviation of the sample by  $\sqrt{n-3}$  instead of the usual  $\sqrt{n}$  in obtaining the standard deviation of the mean of samples. For  $n > 10$ , the value  $z = 0.8$  for the assumption of normality may be increased somewhat. If  $z$  is too large to justify the normal curve when  $n > 10$ , further values may be added to Student's table without great difficulty. For  $n < 4$ , the method has little or no value (see p. 22).

Finally, we wish to call attention to the possibility of applying statistical methods to another important problem in physiology, i.e., the correlation existing between different functions, or between a function and any substance administered, or between a function and a measurable condition of the environment. For example, it is important to know to what extent the excretion of nitrogen as urea, ammonia, creatinin, uric acid, etc., is dependent upon the nitrogen intake, or the weight of dry feces is dependent upon the solid food ingested, or the percentage of the total urinary nitrogen in the form of urea is dependent upon the assimilated nitrogen, etc. Such examples illustrate the problems of correlation.

As the first and simplest method, it may possibly occur that correlation is so pronounced that it may merely be necessary to look at two sets of figures to note that corresponding values have a tendency to change simultaneously in the same or in opposite directions. As a second and somewhat more effective method, one may plot curves for each of the two systems of variates and, if correlation is very pronounced, it may sometimes be discovered by simple inspection that the curves have a tendency to rise and fall together, or that when one rises the other falls.

Not only do these methods prove inadequate to detect correlation unless it is exceedingly pronounced, but they lack precision

in that they do not give a *measure* of correlation. It is not enough to know whether correlation exists; its quantitative measure is usually a matter of importance. Our power to measure correlation between associated phenomena has been enormously increased during the past two decades by methods introduced by Galton and developed by Pearson and those associated with him.<sup>1</sup> In brief we may say that the usual measure of correlation is a single number  $r$ , which is the mean of the products of deviations of corresponding variates from their respective means in units of the standard deviations. With perfect correlation,  $r = \pm 1$ , that is to say, if values are so associated that the ratio of corresponding deviations is constant and positive for all pairs of variates, then  $r = 1$ ; if the ratio is constant and negative,  $r = -1$ . When there is no correlation  $r = 0$ . For other cases,  $r$  assumes values between  $-1$  and  $+1$  and becomes a general measure of correlation which is of much service in statistics and may well be in physiology. The probable error of the coefficient of correlation  $r$  is

$$E_r = 0.6745 \frac{1-r^2}{\sqrt{n}}$$

where  $n$  is the number of correlated pairs.

#### CONCLUSIONS.

1. The occurrence of functional variability, *i.e.*, the residual variability of physiological functions when all conditions, in so far as we are cognizant of and can control conditions, are constant, is universal among living organisms, and the deviations which it effects in quantitative measurements of such functions are often considerable.

2. These deviations whose antecedents we cannot trace, constitute an obstacle, oftentimes a serious obstacle, to the formulation

<sup>1</sup> The theory of correlation is found largely in the memoirs of Karl Pearson in the *Philosophical Transactions of the Royal Society*, under the general title "Mathematical Contributions to the Theory of Evolution." A brief and simple statement of the elements of the theory is given in the Appendix to E. Davenport's "*Principles of Breeding*." The mechanical details of applying the theory can be well obtained from C. B. Davenport's "*Statistical Methods*."

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of legitimate conclusions from data of metabolism and render problems of metabolism statistical in their nature.

3. It is a legitimate hypothesis that the causes of the variations are such that the latter may be dealt with more satisfactorily under certain conditions, especially in connection with results (such as a mean, a standard deviation, a coefficient of correlation, etc.), by the application of the so-called "probable error" obtained from Gauss's exponential equation.

4. Not only is the probable error a convenient criterion for the evaluation of experimental results in metabolism, but it is a *necessary* criterion that must, in many cases, be applied to draw definite, especially quantitative or general conclusions that legitimately be deduced.

5. It follows, therefore, that experiments in this field should be so planned as to conform the most strictly with the requirements for the application of the law of probability and to yield the most valuable results as judged by this method.

6. Under such conditions, we may confidently expect that the laws of probability, together with the various mathematical methods for reducing statistical data in common use among statisticians, will render a tremendous service to physiology and physical chemistry as they have to do with such problems of general biology, heredity and evolution. Further, we do not hesitate to say that, by the use of such methods, a greater degree of exactness and precision and, conversely, a narrower field for the exercise of unaided judgment or biased opinion in the interpretation of experimental data, will result, fully repaying the extra labor and expense involved in conforming to the requirements of these methods.

We wish to acknowledge the valuable assistance, in the form of encouragement and suggestions, which we have constantly received from H. S. Grindley, the chief of this laboratory, as well as the unlimited use of as yet unpublished experimental data.

# THE EFFECTS OF BLEACHING UPON THE DIGESTIBILITY OF WHEAT FLOUR.

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In the studies which have been recently made to determine the effects caused by the action of nitrogen peroxide when it is used in the bleaching of flour the question of a possible alteration of the digestibility of the flour has occupied a prominent place. The methods employed to investigate this differ and the results obtained are conflicting. In the bleaching process, where nitrogen peroxide is the active agent, chemists generally agree that there is added to the flour a substance which gives the nitrite reaction when the Griess-Ilosvay test is applied. Alway<sup>1</sup> found an average of 1.3 parts of nitrite nitrogen per million of flour from analyses of 58 samples of bleached flours, with one as high as 5.5 parts (not as incorrectly quoted by Hale<sup>2</sup> 6.3 and 27 respectively who gives figures for amounts calculated as sodium nitrite.) Only five of the 58 contained over 2.5 parts of nitrite nitrogen per million. This nitrite reacting material has been suspected of modifying the action of the digestive enzymes.

Halliburton<sup>3</sup> tried to get light upon the point by adding small quantities of sodium nitrite to the digesting mixtures. Starch paste was treated with saliva and the time noted when the liquid gave no blue color with iodine (achromic point). In peptic digestion fibrin stained with carmine was used and the speed judged by the depth of red color imparted to the liquid. Halliburton con-

<sup>1</sup> Alway: Bull. No. 102. Agricult. Exp. Station, Univ. of Nebraska.

<sup>2</sup> Hale: Bull. No. 68, Hygienic Laboratory, Treasury Department, Washington, 1910.

<sup>3</sup> Halliburton: *Journal of Hygiene*, ix, p. 2, 1909.

cluded that the nitrite retarded digestion. There is no evidence that the bleached and unbleached flours were the same in composition or starch content and the fact was overlooked that nitrous acid bleaches carmine in acid solution and that therefore the pepsin test is valueless. The digestibility of gluten was tried with pepsin using enough of the moist gluten to correspond to 5 gms. of the dry substance. After 16 hours the undigested residue was dried on a weighed filter. The bleached left a larger residue than the unbleached. Only mean results are given and it is not stated how closely parallel tests agree. The digestibility was not proportional to the nitrite nitrogen in the flour.

Mann<sup>1</sup> found that, tested with saliva, there "was a distinct change induced in the starch as a result of bleaching" if the flour contained more than 4 parts of nitrite nitrogen per million, also that the starch of one flour containing 7 parts of nitrite nitrogen digested more rapidly than another which contained 2.5 per million. Gluten was washed from a pound of flour; that from unbleached flour was more adhesive,—so sticky that it could not be readily removed from the fingers. The gluten was drawn into glass tubes (diameter not stated) and, after steaming, sections of equal length were placed in solutions of pepsin and of pancreatin. More of the unbleached gluten went into solution than of the bleached, as shown by measurements.

Ladd and Bassett<sup>2</sup> tested with a pepsin solution, also with a pancreatin solution, the moist gluten washed out from 10-gm. portions of flour to learn the time at which complete solution took place. "The time could not be accurately determined but when there was marked difference, as shown in some cases, a relative figure could be determined." The bleached gluten was judged to be more slowly digested. Similar results were obtained with baked gluten and with bread from bleached and unbleached flour.

Hale<sup>3</sup> used the method of Ladd and Bassett. When 50 cc. more of nitric oxide were used per kilo of flour, leaving in the flour 6.1 parts of nitrite nitrogen per million, although there was a

<sup>1</sup> Mann: Testimony before Commissioner, Notice of Judgment No. 2 Food and Drugs Act, U. S. Dept. of Agriculture, 1910.

<sup>2</sup> Ladd and Bassett: *This Journal*, vi, p. 75, 1909.

<sup>3</sup> Hale: *loc. cit.*

siderable variation in duplicate tests, the average time of digestion was greater for the bleached than that for the unbleached gluten. When 10 to 30 cc. of nitric oxide per kilo were used, although the average results were similar to those with the heavily bleached gluten, the variations were great and in some cases the unbleached digested more slowly.

On the other hand, Snyder<sup>1</sup> reports with men the same degree of digestibility of bread and, with a pepsin solution, the same amount of insoluble nitrogen from the bleached and unbleached flours.

Wesener and Teller<sup>2</sup> found that pepsin converted to the soluble form slightly more nitrogen from bleached than from unbleached flour.

It therefore seemed desirable to make further tests upon the digestibility of the constituents of wheat flour and bread. The plan was to use for parallel tests the same flour or bread, part of which had been bleached by nitrogen peroxide, part of which had not received this or any other bleaching treatment. For the most part commercially bleached flours were used although, in some cases, the bleaching was carried out in the laboratory. Of the commercial flours the bleached and unbleached from Iowa City are known to be from the same lot of flour. The others were ordered through channels which leave no doubt that they were such. Typical protocols are given.

#### THE DIGESTION OF MOIST GLUTEN.

500–800 gms. of flour, after thoroughly moistening and standing for an hour, were washed with water, while kneading under the nap, until the washwater ran off clear. Still, even after several hours, the iodine test showed the presence of a small amount of starch. The gluten from the unbleached flour was yellow; that from the bleached flour, much more nearly white. The excess of water was removed as much as possible by squeezing and wiping

<sup>1</sup> Snyder: Univ. of Minnesota, Agricultural Experiment Station, Bulletin No. 111, 1908.

<sup>2</sup> Wesener and Teller: *Journal of Industrial and Engineering Chemistry*, p. 700, 1909.

with a towel. After thoroughly kneading, 2-gm. portions were taken to determine the nitrogen content by Kjeldahl's method. 5-gm. portions for digestion. The difficulty of getting a completely homogeneous mass, or portions containing exactly the same amount of water-free gluten, was recognized, the comparatively large water content being responsible for this. The gluten was rolled into balls to expose the same surface to the digestive solution and put into small Erlenmeyer flasks containing the solution of pepsin or pancreatin. If the balls were previously moistened they would not stick to the glass either before or after entering the solution. The flasks stood in pairs, of bleached and unbleached flour respectively, in a water bath at 38°, and were shaken frequently and equally. At the conclusion of the digestion period the soluble portion was poured from the gluten residue; the residue was washed three or four times by decantation and the solution combined with the wash-water was placed in a Kjeldahl flask with a small amount of concentrated sulphuric acid. The water was boiled off, mercuric oxide added, and the resulting liquid digested with concentrated sulphuric acid until the organic matter was destroyed. The ammonia was determined in the usual manner. The nitrogen in the insoluble part of the gluten was found in the same way. The variation in the amount of water in the different portions is indicated by the differences in the total nitrogen in the individual tests, as compared with the differences between the total nitrogen after digestion and that found in the moist gluten before digestion. The coefficient of nitrogen in the original moist gluten, which was the average of two, and sometimes three, determinations, is used only for comparison the percentages of digestible and undigestible gluten being calculated from the nitrogen found in the soluble portion and the residue respectively.

#### *Pepsin-hydrochloric Acid Digestion.*

The gluten was obtained from two flours. Lexington Flour was a Nebraska flour containing 0.91 part of nitrogen in the bleached, none in the unbleached. For the digestion 1 fluid gm. of Merck's pepsin was dissolved in a liter of 1 per cent hydrochloric acid and 75 cc. used with 5 grams of



gluten. The amount of nitrogen contained in the 75 cc. of the gastric juice was found to be only a fraction of a milligram and no correction was made for this.

PEPSIN-HYDROCHLORIC ACID DIGESTION OF MOIST GLUTEN.

Lexington Cream Flour.

*I. Digested one hour and ten minutes.*

	BLEACHED	UNBLEACHED
Soluble nitrogen.....	0.0297 gm. = 13.6 %	0.0276 gm. = 11.6 %
Insoluble nitrogen.....	0.1885 gm. = 86.4 %	0.2095 gm. = 88.4 %
Total.....	0.2182 gm.	0.2371 gm.

*II. Digested two hours and thirty minutes.*

	BLEACHED	UNBLEACHED
Soluble nitrogen.....	0.0417 gm. = 19.2 %	0.0373 gm. = 14.7 %
Insoluble nitrogen.....	0.1755 gm. = 80.8 %	0.2168 gm. = 85.3 %
Total.....	0.2172 gm.	0.2541 gm.

*III. Digested three hours and thirty-five minutes.*

	BLEACHED	UNBLEACHED
Soluble nitrogen.....	0.0631 gm. = 28.0 %	0.0696 gm. = 26.8 %
Insoluble nitrogen.....	0.1639 gm. = 72.0 %	0.1902 gm. = 73.2 %
Total.....	0.2270 gm.	0.2598 gm.

*Calculated nitrogen in five grams of moist gluten.*

Bleached.....0.2125 gm.      Unbleached.....0.2385 gm.

Golden West Flour was from North Dakota and contained 0.57 part of nitrite nitrogen per million in the bleached, none in the unbleached. Five gms. of moist gluten were used for each test with 75 cc. of gastric juice (0.5 gm. of Kahlbaum's pepsin per liter of 0.25 per cent hydrochloric acid).

## Digestibility of Bleached Flour

## PEPSIN-HYDROCHLORIC ACID DIGESTION OF MOIST GLUTEN

Golden West Flour.

*I. Digested one and one-half hours.*

	BLEACHED	UNBLEACHED
Soluble nitrogen.....	0.0494 gm. = 17.9 %	0.0469 gm. = 17.7 %
Insoluble nitrogen.....	0.2258 gm. = 82.1 %	0.2181 gm. = 82.3 %
Total.....	0.2752 gm.	0.2650 gm.

*II. Digested two and one-half hours.*

	BLEACHED	UNBLEACHED
Soluble nitrogen.....	0.0709 gm. = 25.9 %	0.0737 gm. = 27.2 %
Insoluble nitrogen.....	0.2032 gm. = 74.1 %	0.1972 gm. = 72.8 %
Total.....	0.2741 gm.	0.2709 gm.

*III. Digested three and one-half hours.*

	BLEACHED	UNBLEACHED
Soluble nitrogen.....	0.0995 gm. = 35.9 %	0.0916 gm. = 33.3 %
Insoluble nitrogen .....	0.1776 gm. = 64.1 %	0.1836 gm. = 66.7 %
Total.....	0.2771 gm.	0.2752 gm.

*Calculated nitrogen in five grams of moist gluten.*

Bleached.....0.2609 gm.      Unbleached.....0.2705 gm.

The gluten from the Lexington flour appears somewhat more digestible after bleaching than before; that from Golden West flour shows practically no difference. It would seem to be a fair conclusion that moist gluten from bleached flour is at least no less readily digestible by pepsin than where the flour has not undergone the bleaching process.

Pancreatic Digestion.

Five gms. of moist gluten from Lexington flour (from the same lot as the last) were used with 75 cc. of pancreatic juice (1 gm. of Merck's pancreatin in 600 cc. of water, faintly alkaline with sodium bicarbonate). There was 0.012 gm. of nitrogen in the 75 cc. of the juice used and this amount was subtracted from the nitrogen of the filtrate before calculating the percent.

PANCREATIC DIGESTION OF MOIST GLUTEN.

Lexington Cream Flour

I. Digested one hour.

	BLEACHED	UNBLEACHED
Soluble nitrogen.....	0.0043 gm. = 1.7 %	0.0069 gm. = 2.8 %
Insoluble nitrogen.....	0.2393 gm = 98.3 %	0.2371 gm. = 97.2 %.
Total.....	0.2436 gm.	0.2440 gm.

II. Digested four hours.

	BLEACHED	UNBLEACHED
Soluble nitrogen.....	0.0257 gm. = 10.5 %	0.0214 gm. = 8.5 %
Insoluble nitrogen.....	0.2182 gm. = 89.5 %	0.2306 gm. = 91.5 %
Total.....	0.2439 gm.	0.2520 gm.

III. Digested five hours.

	BLEACHED	UNBLEACHED
Soluble nitrogen.....	0.0351 gm. = 14.5 %	0.0293 gm. = 11.6 %
Insoluble nitrogen.....	0.2066 gm. = 85.5 %	0.2238 gm. = 88.4 %
Total.....	0.2417 gm.	0.2531 gm.

Calculated nitrogen in five grams of moist gluten.

Bleached.....0.2450 gm.      Unbleached.....0.2483 gm.

The gluten from the unbleached flour appears to be somewhat less digestible by trypsin than that from the bleached.

DIGESTION OF COOKED GLUTEN.

The gluten was extracted by washing the flour as before, then cooked by heating on a steam bath several hours, dried at 100°, and ground by passing it several times through a coffee grinder, set as closely as possible. The digestions were carried on in small Erlenmeyer flasks which stood in a water bath at 38°. The flasks containing the bleached and unbleached gluten always stood side by side in pairs and were shaken equally at the same time. At the expiration of the time of digestion the contents were filtered, well washed with water and the nitrogen of the insoluble residue (which was regarded as the quantity undigested) was determined by treating the residue with the filter according to Kjeldahl's method. The difference between the original nitrogen content, also determined by Kjeldahl's method, and that thus recovered was called the amount digested. The nitrogen in the solution was not determined, as was done with the moist gluten inasmuch the water had been removed from the gluten by long drying at 100° and its nitrogen content could therefore be accurately determined.

PEPSIN-HYDROCHLORIC ACID DIGESTION.

*Gluten from Lexington Cream Flour.*

0.91 parts of nitrite nitrogen per million in the bleached flour; none in the unbleached.

1 gm. of Kahlbaum's pepsin was dissolved in a liter of 0.2 per cent hydrochloric acid; 80 cc. of this were used with 2 grams of the dried gluten.

AMOUNT DIGESTED AT THE END OF	BLEACHED	UNBLEACHED
30 minutes.....	67.3 per cent	62.4 per cent
1 hour.....	73.6 per cent	72.1 per cent
2 hours.....	91.7 per cent	89.1 per cent

Steamed and dried gluten from bleached flour digested somewhat more rapidly than that from unbleached.

PANCREATIC DIGESTION.

Lexington flour was used, as in the pepsin digestion. One gram of Merck's pancreatin was dissolved in 500 cc. of water, enough sodium carbonate added to make it faintly alkaline and 75 cc. used, with grams of dried gluten, for each test.

AMOUNT DIGESTED AT THE END OF	BLEACHED	UNBLEACHED
10 minutes.....	10.6 per cent	9.4 per cent
1 hour and 30 minutes....	26.4 per cent	22.0 per cent
2 hours and 30 minutes....	38.5 per cent	32.0 per cent

Iowa City flour was used to prepare gluten for the following two tests. The bleached contained 2.64 parts per million of nitrite nitrogen; the unbleached contained none. The gluten was prepared, dried and ground as above. Seventy-five cc. of pancreatin solution were used for each digestion (1 gm. of Merck's pancreatin per liter). In the first and second 1.0 gm. of dry gluten was used; in the third 1.5 gm. and this, after drying and previous to digestion, was boiled with considerable water in an attempt to remove the small amount of starch which it held and thereby facilitate subsequent filtration. After three hours 5 cc. of a rather concentrated pancreatin solution was added to the bleached and the unbleached gluten of the second test. The undigested residue was filtered off, washed and its nitrogen determined by Kjeldahl's method, the difference between this and the original being assumed to represent the digestible nitrogen.

	AMOUNT DIGESTED AT THE END OF	BLEACHED	UNBLEACHED
I	2 hours, 10 minutes.....	61.4 per cent	57.9 per cent
II	3 hours, 50 minutes.....	75.0 per cent	73.1 per cent
III	2 hours, 10 minutes.....	86.4 per cent	86.1 per cent

DIGESTIBILITY OF BREAD.

The bread was made with yeast under the usual domestic conditions and baked in a coal range. After it had become dry it was finely ground with a coffee grinder and well mixed before sampling. Only the digestibility of the protein was tried.

*Pepsin-hydrochloric Acid Digestion.*

The bread was made from Iowa City flour which contained 2 parts of nitrite nitrogen per million (bleached); the unbleached flour showed no reaction for nitrites. The bread made from the bleached flour contained no nitrites when taken from the oven. 2 gms. of the bread were used with 75 cc. of gastric juice (1 gm. pepsin per liter of 0.2 per cent hydrochloric acid). After washing the nitrogen was determined by Kjeldahl's method in the undigested residue and the difference between that and the original was regarded as the measure of digestion. A considerable difficulty was experienced in filtering and washing, because of the presence of the starch.

AMOUNT DIGESTED AT THE END OF	BLEACHED	UNBLEACHED
50 minutes.....	70.8 per cent	67.2 per cent
2 hours and 5 minutes ..	79.5 per cent	79.2 per cent
2 hours and 45 minutes..	82.9 per cent	86.4 per cent

The conclusion is that the protein of bread made from bleached flour digests as well as that from unbleached.

THE DIGESTION OF STARCH.

*Pancreatic Digestion.*

Minneapolis flour was used, a sample of commercially bleached being compared with some of the same flour which had not been bleached. The bleached flour contained 0.6 part per million nitrite nitrogen; the unbleached was free from it. The degree of digestion was determined (I) from the amount of reducing sugar formed in a given time and (II) from the time which elapsed before the iodine reaction would fail to appear.

I. Ten gms. of flour were moistened, then boiled with a large amount of water and the liquid diluted to 1000 cc. Twenty cc. of this were used with 5 cc. of pancreatin solution (0.5 gm. of Merck pancreatin in 100 cc. of water). After standing 30 minutes

was diluted to 50 cc. and run into 5 cc. of hot Fehling's solution. The same amount was required of the solution from bleached and the unbleached flour to destroy the blue color—

c.  
The same solution of flour as the last was used, 20 cc. being used for each digestion; equal amounts of iodine in potassium solution were added to each tube after the addition of the pancreatin solution (0.5 gm. per liter). All the tubes were digested in the same water bath at 38°. As the digestion proceeded very slowly, after 1 hour and 20 minutes, 3.5 cc. more of the pancreatin solution were added to the first pair of tubes and 3.0 cc. to each of the others. The time until the achromic point was reached was the same for each pair of tubes, bleached and unbleached, and the speed was the same as shown by their having the same shades of purple and as the erythrodextrin was formed and destroyed. The amount of pancreatin solution used for each 20 cc. of flour solution and the time of reaching the achromic point are given below.

FLOUR SOLUTION	TIME	PANCREATIN SOLUTION	TIME
+ 3.5cc.	1 hour, 39 minutes	4 cc. + 3 cc.	42 minutes
- 3 cc.	1 hour, 35 minutes	5 cc. + 3 cc.	31 minutes
- 3 cc.	1 hour, 32 minutes		

It was found that very slight variations in treatment of the two of a pair would cause very considerable differences in the results but with identical modes of manipulation the time at which the color disappeared was the same. For instance, the amount of iodine present markedly altered the time of disappearance of the blue and red; this appears from the following series.

DROPS OF IODINE SOLUTION	TIME OF REACHING ACHROMIC POINT	
	Bleached	Unbleached
5	2 minutes	2½ minutes
6	4½ minutes	4½ minutes
7	5½ minutes	5½ minutes
8	5½ minutes	7½ minutes
10	14½ minutes	13½ minutes

Lexington flour was used, 4 gms. being boiled and diluted to a liter. An equal volume of pancreatin (0.5 gm. in 200 cc.) solution was taken for each portion, the whole being made slightly alkali with sodium bicarbonate.

The iodine was not measured but dropped from an ordinary dropping bottle which as is well known does not always deliver the same sized drops. In these tests also the starch from the bleached flour appears to be saccharified as rapidly as that from the unbleached.

#### *Salivary Digestion.*

This was tested with boiled flour and saliva at 38°, adding iodine in potassium iodide solution immediately after the saliva. This was found to be an advantage as the digestion, indicated by the gradual change of color, could be followed more readily. It was compared with the method by removing small portions to test with iodine, and the latter was found to be in many ways less satisfactory while showing no different results.

FLOUR USED	NITRATE NITROGEN PER MILLION	WEIGHT PER LITER OF WATER	VOLUME USED		WATER ADDED	SALIVA	IODINE SOLUTION	ACROMATIC BY REACTOR	
								Bleached	
		gms.	cc.	cc.	cc.	cc.	cc.	min.	sec.
Bleached in laboratory	1.00	10	20	—	0	(?)	■	38	
			20	—	4	(?)	■	43	43
			20	—	5	(?)	■	46	46
Chaffee-Miller	0.76	10	10	—	5	(?)	■	22	22
			10	—	3	(?)	■	43	43
Lidgerwood	0.64	10	10	—	10	(?)	■	9	■
Golden West	0.57	10	4	10	5	0.5	■	3.75	3
			10	10	5	0.5	■	3.7	3
			20	—	4	0.3	■	15.0	15
Iowa City	2.64	4	10	■	■	0.1	■	9.5	9
			20	—	4	0.5	■	16.0	16
Lexington	0.01	4	20	—	3	10 drops	■	7.5	7
			20	—	2	5 drops	■	4.8	4
			20	—	5	15 drops	■	11.5	11
			30	—	3	8 drops	■	5.0	5



Some of the tests were made at different times and with different specimens of saliva and different concentrations of iodine, so that only the members of the corresponding pairs are strictly comparable. The pairs were always made together and under the same conditions. No evidence appears that the ptyalin digests the starch of bleached flour more slowly than that of unbleached flour. This is shown not only by the achromic point being reached after the same interval but by the two solutions having the same shade of color as digestion progresses.

### *Digestion by Diastase.*

Unbleached flour was bleached in the laboratory by shaking in a flask with nitrogen peroxide. It then contained one part of nitrite nitrogen per million: there was none in the unbleached. Ten gms. of the flour were thoroughly boiled with a liter of water, the liquid filtered, and 20 cc. of the bleached and the unbleached flour solutions were treated with the same volume of a diastase solution (5 gms. of Merck's diastase per liter of water) and kept at 38,° a few drops being removed at intervals and tested with iodine. The rate of digestion was the same as shown by the shade of color obtained.

### SUMMARY.

Studies upon the digestibility of the constituents of wheat flour which has been bleached by nitrogen peroxide have given conflicting results.

The tests reported here were made upon a number of samples, a bleached and an unbleached sample from the same lot of flour being run at the same time and in the same manner.

Moist, uncooked gluten was tested with pepsin-hydrochloric acid and that from bleached flour was found to digest as rapidly, and in some cases more rapidly, than that from unbleached flour.

Moist gluten was also digested with a pancreatin solution and that from bleached flour digested more readily than that from unbleached flour.

Gluten from bleached flour, after it had been steamed and dried, digested somewhat more rapidly than that from unbleached flour. This was true with either the pepsin or the pancreatin solution.

Bread made with yeast from bleached flour did not differ in digestibility from that from unbleached. The nitrite reacting material largely or altogether disappears before the bread is removed from the oven.

Boiled starch prepared from bleached and unbleached flour forms, by the action of pancreatin, reducing sugar at equal speeds. Tested with iodine there is no difference in the rapidity of starch digestion, either by pancreatin or by the salivary ferment. Diastase gives the same result.

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# STUDIES ON MELANIN.

## I. METHODS OF ISOLATION.

### THE EFFECT OF ALKALI ON MELANIN.

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## I. INTRODUCTION.

1. *The Problem.* In undertaking a study of animal pigmentation, the author was impressed by the many conflicting statements relating to the behavior of substances of the melanin class. No one, apparently, having undertaken a study of sufficient breadth to determine the true nature of these bodies, it was deemed advisable to expend considerable time in preliminary researches, in order to ascertain the behavior of these pigments towards those reagents which are ordinarily used to extract or purify the coloring matter.

The data recorded in this article relate to the action of caustic alkali upon the melanin, caustic alkalies having been very generally used to destroy the keratin structures and to dissolve the pigment. By a series of experiments in which the strength of alkali was gradually increased, it has been possible to obtain a series of products, the detailed study of which serves to show the decomposing effect of alkali.

2. *Nomenclature.* By the term "melanin" the author wishes to designate only those dark pigments which occur normally or pathologically in the animal body, skin, hair, or feathers. It has been generally assumed that these bodies are all insoluble in indifferent solvents and in dilute acids, but, as will be shown later, this definition will not always hold true.

Many authors refer to "melanin," "artificial melanin," or "melanotic substances" in speaking of the black humic substances resulting from the hydrolysis of proteins by strong mineral acids (Schmiedeberg (1897), Chittenden and Albro (1899), Samuely (1902), and others), or those dark products formed by the action of oxydases upon aromatic or heterocyclic phenols (Bertrand (1896), von Fürth and Jerusalem (1907), von Fürth and Schneider (1902), etc.). It may, sometime, be shown that these substances are related to the melanins, but until that relationship is demonstrated, they should not be confused with the true animal pigments. Osborne and Jones (1910) very rightly prefer to call these artificial compounds, "humins."

3. *Historical.* It will be impossible to include in this paper a complete summary of the work which has been done in the field of the melanin compounds. Only a few typical researches, selected as the most important for the present purpose, will be reviewed

here, the reader being referred to von Fürth's (1904) bibliography of the melanin work for additional references.

Abel and Davis (1896), in a study of the pigment of the negro's hair and skin, found that the "pigment-granules" could be separated from the keratin by heating at  $100^{\circ}$  in a nickel dish with a 5-6 per cent solution of potassium hydrate. The heating was continued until all the keratin had dissolved. The mixture was allowed to cool, filtered, and the "pigment-granules" (pigment and pigment structure) dried and washed with warm hydrochloric acid, alcohol, chloroform and ether. As an alternate method they state that the alkali may be replaced by concentrated hydrochloric acid.

In order to free the pigment from the pigment structure the granules were treated with 5 per cent hydrochloric acid for ten days, decanting and renewing acid every two days, then treated with warm dilute potassium hydrate until solution took place, whereupon an equal volume of water was added and the whole filtered. The pigment was precipitated by the addition of six volumes of alcohol and one of ether, let settle, decanted, washed with alcohol acidified with acetic acid, dissolved in potassium hydrate solution, and the entire process repeated seven or eight times. The pigment was finally dried and powdered.

Landolt (1899), studying the melanin of the eye coat, removed the granules by rubbing them loose under water, precipitating the pigment with saturated ammonium sulphate solution, washing with water, drying and extracting with ether and alcohol in a Soxhlet apparatus. Landolt makes a very significant statement when he adds, "when boiled with acid the pigment is modified, the nitrogen content being reduced, and, of course, the oxygen content being increased."

Von Fürth and Jerusalem (1907) studied the pigments formed in melanotic tumors, the pigment being prepared by first "boiling the tumors for some hours with fuming hydrochloric acid until only the pigment granules remained undissolved," filtering, washing, again boiling with fuming hydrochloric acid, filtering, washing again, repeating the process and, lastly, extracting with alcohol and ether. They describe their product as "insoluble in hot concentrated acid, hot concentrated alkali or any ordinary solvents."

Mörner in 1887 published an extensive study on "The Coloring

Matter of Melanotic Tumors," some parts of which are of extreme interest. The source of the melanin was the urine of man who subsequently died of melanotic tumors. In isolating the melanin the urine was precipitated with baryta, and the collected precipitates washed in a tall cylinder by decantation, filtered and pressed out. The precipitate was decomposed with strong soda solution in which the greater part of the pigment dissolved. This solution was filtered, precipitated with dilute sulphuric acid, filtered, dissolved in dilute sodium hydrate and an excess of acetic acid added. This caused a portion of the pigment to be precipitated while *a part remained in solution*. The precipitate was purified by repeated solution in dilute sodium hydrate and precipitation with acetic acid, extracted with alcohol and ether and dried. The pigment, soluble in acetic acid, was precipitated with baryta, washed with alcohol and ether and dried. Mörner describes the pigment soluble in acetic acid as "a brown-black amorphous powder, not melting below 120°, insoluble in indifferent solvents or in dilute acids, readily soluble in ammonia, alkaline sodium carbonate or a dilute solution of di-sodium phosphate. He further states (page 96) that "the solution in di-sodium phosphate can be acidified with hydrochloric acid to strong acid reaction without causing precipitation, the addition of a large quantity of acid causing the pigment to be thrown down." From this statement it would appear that his pigment was *not* insoluble in dilute mineral acids *providing the acid was sufficiently dilute*.

The only other research which will be mentioned is that of Eduard Spiegler (1903) who investigated the melanin of black sheep's wool and black horsehair. Spiegler's work is of especial interest, inasmuch as the observations recorded in this paper were also made upon the melanin of black sheep's wool and therefore his results should be directly comparable with mine.

Spiegler claims to have isolated a "white melanin" from white wool and white horsehair. I (1910) have elsewhere shown that this white product, which occurs in small amounts compared with the black pigment, is, probably, a decomposition product of the keratin in that it is found not only in animal plumage and hair where the color is "dominant"<sup>1</sup> (sheep, white leghorn fowl, etc.,

<sup>1</sup> Used in the Mendelian sense.

but also in those instances where the color is "recessive" (albinos), and also in horn structure. A more complete chemical study of this product, is in progress.

In the preparation of the melanin, Spiegler seems to have followed, to some extent, the method of Abel and Davis (1896):

The black sheep's wool was washed with 0.5 per cent sodium carbonate solution and then boiled with 5 per cent potassium hydrate solution (5 liters alkali to 1 kilo wool) for four hours when solution was apparently complete. Concentrated hydrochloric acid was then added to strong acid reaction, the liquid decanted from the precipitate, the precipitate filtered, washed, and then boiled for eight hours with 5 per cent hydrochloric acid. This left a fine brown powder which was filtered off, dried on a water bath, dissolved in dilute ammonia, filtered, precipitated with hydrochloric acid, washed, dried, powdered, dissolved in concentrated sulphuric acid, precipitated by dilution, washed, dried, again dissolved in concentrated sulphuric acid, filtered through glass wool, precipitated, washed, dried, washed with alcohol, carbon disulphide and ether, and again dried. The resulting product was a dark black powder with an ash content of 10.85 per cent.

The analytical data will be given later in comparison with the author's figures.

4. *A Discussion of the Historical Data; the Chemical Behavior of Melanin:* As can readily be seen by a study of the historical data, the class of the melanin compounds has been regarded as very inert chemically. Hoppe-Seyler (1909) characterizes the group as non-crystalline, granular, insoluble in all indifferent solvents and in dilute acids, more or less soluble in alkalis *and very resistant to powerful chemical reagents*. That these statements have been very generally accepted may readily be seen in that Abel and Davis (1906) state that concentrated hydrochloric acid may replace 6 per cent caustic alkali in the isolation of the melanin. Spiegler (1903) used 5 per cent caustic alkali and afterwards boiled for eight hours with 5 per cent hydrochloric acid, although in his later article (1907) he "purified" the melanin, prepared from black horse hair, by boiling for nine hours with fuming hydrochloric acid.

Von Fürth and Jerusalem (1907) have, if possible, used more drastic treatment, boiling both with fuming acids and concentrated alkalies, after which treatment their characterization of melanins must, necessarily, be true that they "are insoluble in indifferent solvents and in acid media."

It seemed impossible to me that any animal product could be wholly indifferent to the action of strong caustic alkali, or

fuming hydrochloric acid, and this supposition has been found to be true in the case of melanin from black wool. As will be shown later in this paper, melanins are very readily decomposed by caustic soda solutions when the strength of the alkali exceeds 0.2 per cent. In a later article it will also be shown that strong mineral acids readily decompose the melanin bodies, boiling with 25 per cent sulphuric acid, splitting off in some instances as much as 90 per cent of the pigment. I have found that a *black product can be prepared from black wool by almost any method*, but, in order to prepare the *melanin, i.e., the pigment, in the form which exists in the living animal*, great care is needed to prevent decomposition.

Landolt (1899) seems to have recognized the decomposing effect of acids, for he states that when he boiled his melanin with concentrated hydrochloric acid, filtered, and evaporated the filtrate to dryness, he obtained "tyrosin-like crystals," although in too small a quantity for identification with certainty, although they gave Millon's reaction. I have obtained similar results with the melanin from black wool, and, although the crystals are not completely identified they give both Millon's reaction and the typical tyrosin coloration in the presence of tyrosinase.

Among those pigments already referred to, the one prepared by Mörner, which was found to be soluble in acetic acid, seems to the author to have been most nearly pure. Even with this pigment, however, Mörner states that *barium was present in the ash* (ash 4.00 per cent), the pigment having been precipitated by barytes. From this fact I believe that Mörner worked with *the barium salt of the melanin*, for a melanin is readily obtained from black sheep's wool which is soluble in acetic acid, soluble in very dilute hydrochloric acid, contains *no ash*, and readily forms stable metallic salts.

In a later article will be given a detailed description of the metallic salts, of the products of the acid hydrolysis of the melanin and of compounds which are readily formed by the action of organic reagents upon the pigment, such as phenyl hydrazones, etc.

In summing up the chemical nature of melanins, I believe that some melanins are quite active chemically and are readily decomposed. It appears very probable that a great majority of the work recorded on the melanins is really data of melanin decomposition products.



5. *The Source of the Melanin:* The material employed in this research was black sheep's wool, the sheep being adult-hybrids of Downs and Dossets. The wool was washed with hot 0.5 per cent sodium carbonate solution, rinsed with 0.25 per cent soda solution, then thoroughly washed and air dried.

6. *Methods of Analysis of the Purified Pigment:* In the analysis of the purified products the usual methods were employed. The ash content was determined by burning a weighed amount of the pigment in a platinum crucible. The carbon and hydrogen percentages were determined by combustion in a current of oxygen, the tube being filled with fused lead chromate. The nitrogen was determined by the Dumas method. The sulphur content was found by fusing a weighed amount of the pigment in a nickel crucible with sodium-potassium carbonate and sodium peroxide according to the method of Hoehnel-Glaser modified by Neumann-Meinertz (1904). All percentages are based upon ash-free pigment.

## II. EXPERIMENTAL.

### 7. *Extraction with 0.2 per cent Solution of Sodium Hydrate*

160 grams of black wool were boiled for three hours with 3 liters of 0.2 per cent sodium hydrate solution in a 5 liter Jena flask, using a return condenser. The mixture was then filtered and the residue again boiled in a like manner, filtered, and the process repeated until all of the wool had been dissolved. This gave five filtrates of approximately 3 liters each. Each filtrate was precipitated by the addition of 100 cc. of concentrated hydrochloric acid. The supernatant liquid was removed with a syphon, the precipitate washed by decantation with 6 liters of 1 per cent hydrochloric acid, and then heated just to boiling with one liter of  $\frac{N}{10}$  hydrochloric acid. The precipitates from filtrates 1, 2 and 3 completely dissolved in the dilute acid giving deep black solutions. These solutions were united, filtered, precipitated by the addition of hydrochloric acid equal to 1 per cent, and the supernatant liquid syphoned off. The precipitate was dissolved in 300 cc. of 50 per cent acetic acid and dialyzed against distilled water until free of acid and chlorides. The pigment was now found to be precipitated as a brown flocculent mass. This was filtered off, dried on a water bath, powdered, dried at 110° and extracted successively in a Soxhlet apparatus with carbon disulphide, alcohol and ether. Nothing was removed by any of these solvents. Weight, 13.0 grams or 8.1 per cent of the air dry wool.

When freshly precipitated the pigment is very plastic and may be molded into very compact balls. When this mass is warmed

on a water bath, water is forced out of the contracting mass and becomes very tough, resembling a high grade bitumen: perfectly dry the mass is very tenacious but when dry it readily, the fracture resembling that of a lignite.

Prepared in this manner the melanin is a jet black, mass powdering to a very dark brown dust. When dried the pigment is insoluble in all solvents excepting alkali, which is only slowly and incompletely soluble in alkali. When heated at room temperature it slowly, but completely, dissolves in hydrochloric acid. When moist, solution in alkali takes place almost instantly as does solution in dilute or anhydrous hydrochloric acid, dilute or glacial acetic acid and in hydrochloric acid dilutions equal to  $\frac{N}{15}$  or less, or in other mineral acids of similar concentration. The pigment is also readily soluble in sodium disodium phosphate. From the solutions in acids or alkali the pigment is readily precipitated by the addition of hydrochloric acid equal to 1 per cent, or by half saturation with ammonium sulphate. When boiled with caustic alkalies in concentration exceeding 0.2 per cent the melanin is very readily destroyed, yielding ammonia, alkali sulphides and a pigment insoluble in dilute acids.<sup>1</sup>

When heated the melanin fuses, somewhat, at a high temperature, giving the characteristic odor of burning nitrogenous bodies, and eliminating pyrrol vapors. The pyrrol vapors were observed in all the products studied, but this melanin was the only one in which partial fusion was noticed.

The melanin is easily destroyed by heating with strong acids, giving an insoluble brownish-black powder and a series of products of hydrolysis which will be reported in a later paper.

I have also observed that horse hair (see Spiegler's work) contains an acid-soluble pigment, the study of which will be reported later.

#### ANALYSIS:

Sample I was prepared as above, by dialysis of an acetic acid solution.

Sample II was prepared several months later than sample I, from a different sample of wool by precipitating the acetic acid solution with

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<sup>1</sup> By boiling 2 grams of the melanin for 30 minutes with 5 per cent soda solution only 0.87 gram of "pigment" was recovered, showing a loss of 56 per cent.

chloric acid, dissolving in in  $\frac{N}{10}$  hydrochloric acid and dialyzing this preparation.

*Neither sample contained any ash.* As far as the author is aware, this is the first ash-free melanin recorded.

- I. 0.3667 gm. gave 0.0362 gm.  $\text{BaSO}_4$  = 0.00497 gm. S.
- II. 0.4247 gm. gave 0.0410 gm.  $\text{BaSO}_4$  = 0.00563 gm. S.

- I. 0.1312 gm. gave 16.0 cc. N at  $26^\circ$  and 763 mm.
- II. 0.1010 gm. gave 12.1 cc. N at  $23.5^\circ$  and 761 mm.

- I. 0.1642 gm. gave 0.3177 gm.  $\text{CO}_2$  and 0.1069 gm.  $\text{H}_2\text{O}$ .
- II. 0.1248 gm. gave 0.2403 gm.  $\text{CO}_2$  and 0.0811 gm.  $\text{H}_2\text{O}$ .

*Found:*

Sulphur	= 1.35 and 1.32 per cent.	Average = 1.33 per cent
Nitrogen	= 13.59 and 13.46 per cent.	Average = 13.52 per cent
Hydrogen	= 7.29 and 7.28 per cent.	Average = 7.28 per cent
Carbon	= 52.70 and 52.51 per cent.	Average = 52.60 per cent
	Oxygen (by diff.)	= 25.29 per cent

Calculated for  $(\text{C}_{108}\text{H}_{172}\text{N}_{22}\text{SO}_{38})_x$

C = 52.57: H = 7.28: N = 13.43: S = 1.33: O = 25.39.

In preparing large amounts of this pigment it is advisable, as a time-saving measure, to discard the residue remaining after the second extraction and begin again with new material. In this manner a relatively large amount of the melanin can be obtained without the delay caused by slow filtration which occurs when the keratin structure becomes very finely divided. The long dialysis can also be dispensed with by careful neutralization of the acid solution. The pigment can then be filtered out and washed on the filter with water. The pigment prepared by this method, however, usually contains a few tenths of a per cent of ash, due to imperfect washing.

The precipitates from filtrates 4 and 5 were found to be insoluble in  $\frac{N}{10}$  hydrochloric acid or in acetic acid. After heating just to boiling with  $\frac{N}{10}$  hydrochloric acid, the pigment was allowed to settle, the supernatant liquid syphoned off and the pigment dissolved in cold 0.2 per cent sodium hydrate solution,<sup>1</sup> filtered, precipitated

<sup>1</sup> Dilute sodium hydrate was used instead of ammonia (see Spiegler, 1903) because in case any alkali were absorbed by the pigment the fixed alkalis would appear only in the ash while ammonia would affect the nitrogen determinations.

with hydrochloric acid, washed, dissolved in 0.2 per cent sodium hydrate solution, precipitated, washed free of acid, dried on water bath, powdered, washed thoroughly on a suction filter with hot water, dried at  $110^{\circ}$  and extracted with carbon disulphide, alcohol and ether in a Soxhlet apparatus and again dried at  $110^{\circ}$ . Yield, 2.19 grams, or 1.36 per cent of the air-dry wool.

This pigment forms black granules powdering to a brown dust, insoluble in indifferent solvents and in acid media, easily soluble in alkalis when moist, less readily soluble after drying. The pigment readily forms stable metallic salts.

It is impossible to state whether or not this pigment is present in this form in the wool, for I have found that the more carbon that is taken in the extraction, the greater is the yield of the insoluble pigment and correspondingly less is the yield of the acid-soluble product. In a recent instance the yield of the latter was reduced to 0.9 per cent, but unfortunately the soluble pigment was not weighed. It is probable that this product is a decomposition product of the acid-soluble melanin.

#### ANALYSIS:

0.1465 gm. gave	0.0008 gm. ash.
0.1500 gm. gave	0.0008 gm. ash.
0.4474 gm. gave	0.0379 gm. $\text{BaSO}_4$ = 0.00518 gm. S.
0.2070 gm. gave	19.2 cc. N at $26^{\circ}$ and 768 mm.
0.1885 gm. gave	17.4 cc. N at $24^{\circ}$ and 765 mm.
0.1500 gm. gave	0.2920 gm. $\text{CO}_2$ and 0.0770 gm. $\text{H}_2\text{O}$ .
0.1658 gm. gave	0.3237 gm. $\text{CO}_2$ and 0.0869 gm. $\text{H}_2\text{O}$ .

#### Found:

Ash = 0.54 and	0.53 per cent.	Average	= 0.54 per cent
Ash-free sulphur	= 1.16 per cent.	Average	= 1.16 per cent.
Ash-free nitrogen	= 10.41 and 10.48 per cent		= 10.44 per cent.
Ash-free hydrogen	= 5.78 and 5.85 per cent		= 5.81 per cent.
Ash-free carbon	= 53.37 and 53.52 per cent		= 53.44 per cent.
Oxygen (by diff.)			= 29.15 per cent.

When the acid-soluble melanin is boiled for several days with 0.2 per cent sodium hydrate solution a portion is found to be soluble in  $\frac{N}{10}$  hydrochloric acid. The major part, however, is no longer soluble in acid and after purifying by dissolving in 0.2 per cent sodium hydrate solution, precipitating with hydrochloric acid

washing free of acid, drying, washing with hot water and extracting with alcohol, carbon disulphide and ether it gave the following figures:

**ANALYSIS:**

0.1342 gm. gave	0.0013 gm. ash.
0.1054 gm. gave	0.0010 gm. ash.
0.4138 gm. gave	0.0369 gm. BaSO <sub>4</sub> = 0.00506 gm. S.
0.1319 gm. gave	11.0cc. N at 24° and 762 mm.
0.2378 gm. gave	19.8cc. N at 24° and 765 mm.
0.1768 gm. gave	0.3487 gm. CO <sub>2</sub> and 0.0876 gm. H <sub>2</sub> O.
0.1433 gm. gave	0.2833 gm. CO <sub>2</sub> and 0.0696 gm. H <sub>2</sub> O.

**Found:**

Ash = 0.97 and 0.96 per cent.	Average = 0.96 per cent.
Ash-free sulphur = 1.23 per cent.	Average = 1.23 per cent.
Ash-free nitrogen = 9.46 and 9.50 per cent.	Average = 9.48 per cent.
Ash-free hydrogen = 5.60 and 5.49 per cent.	Average = 5.54 per cent.
Ash-free carbon = 54.31 and 54.44 per cent.	Average = 54.37 per cent.
Oxygen (by diff.)	= 29.38 per cent.

This analysis, it will be noticed, is very similar to that of the "insoluble" melanin, a fact which serves to bear out the belief that the insoluble pigment is a decomposition product of the acid-soluble pigment.

**8. Extraction with a 1 per cent Solution of Sodium Hydrate.**

Three hundred grams of air-dry black wool were boiled for four hours with 3 liters of 1 per cent sodium hydrate solution in a 5-liter "Jena" flask provided with a return condenser. The solution was then filtered through cheese cloth and poured into 7 liters of water containing 350 cc. of concentrated hydrochloric acid. Hydrogen sulphide was evolved and a precipitate rapidly formed. The precipitate was allowed to settle, the supernatant liquid was decanted and the precipitate washed by decantation with 10 liters of 1 per cent hydrochloric acid. The moist precipitate was then heated to boiling with 2 liters of  $\frac{N}{16}$  hydrochloric acid. No pigment dissolved. The pigment was allowed to settle, the acid was decanted and the pigment washed with distilled water by decantation. The melanin was then dissolved in a 0.2 per cent solution of sodium hydrate, filtered, precipitated by acidification with hydrochloric acid, washed by decantation, again dissolved in a 0.2 per cent solution of caustic soda, filtered, precipitated by hydrochloric acid, washed free of acid, dried on a water-bath, powdered, washed on a suction filter with hot water, dried, extracted in a Soxhlet apparatus with carbon disulphide, alcohol and ether, and dried at 110°. Yield, 9.86 grams, or 3.29 per cent of the air-dry wool.

Prepared in this manner the pigment forms brownish-black granules, powdering to a dark brown dust, insoluble in indifferent solvents or in acids, readily soluble in alkalies when moist and less readily soluble after drying.<sup>1</sup>

**ANALYSIS:**

0.2608 gm. gave 0.0022 gm. ash.

0.2008 gm. gave 0.0015 gm. ash.

0.5536 gm. gave 0.0411 gm.  $\text{BaSO}_4$  = 0.00564 gm. S.

0.5138 gm. gave 0.0404 gm.  $\text{BaSO}_4$  = 0.00555 gm. S.

0.2100 gm. gave 19.2 cc. N at 23.5° and 767 mm.

0.2440 gm. gave 21.9 cc. N at 23.0° and 762 mm.

0.1571 gm. gave 0.2984 gm.  $\text{CO}_2$  and 0.0903 gm.  $\text{H}_2\text{O}$ .

0.1522 gm. gave 0.2890 gm.  $\text{CO}_2$  and 0.0910 gm.  $\text{H}_2\text{O}$ .

**Found:**

Ash	=	0.84 and 0.74 per cent.	Average	=	0.79 per cent.
Ash-free sulphur	=	1.02 and 1.09 per cent.	Average	=	1.06 per cent.
Ash-free nitrogen	=	10.46 and 10.22 per cent.	Average	=	10.34 per cent.
Ash-free hydrogen	=	6.49 and 6.75 per cent.	Average	=	6.62 per cent.
Ash-free carbon	=	52.20 and 52.19 per cent.	Average	=	52.20 per cent.
Oxygen (by diff.)				=	29.78 per cent.

**9. Extraction with a 2.5 per cent Solution of Sodium Hydrate.**

Three hundred grams of black wool were boiled for four hours with 2 liters of 2.5 per cent sodium hydrate solution. The solution was then filtered through cheese cloth and poured into 8 liters of water containing 350 cc. of concentrated hydrochloric acid. The precipitated pigment was purified as above. The same general description will also apply. Yield, 8.95 grams, or 2.98 per cent of the air-dry wool.

**ANALYSIS:**

0.2237 gm. gave 0.0019 gm. ash.

0.2718 gm. gave 0.0023 gm. ash.

0.5184 gm. gave 0.0394 gm.  $\text{BaSO}_4$  = 0.00541 gm. S.

0.5230 gm. gave 0.0404 gm.  $\text{BaSO}_4$  = 0.00555 gm. S.

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<sup>1</sup> This product, as well as all melanins extracted by stronger concentrations of alkali, is probably contaminated to a small extent (about 2 per cent) with the product supposedly formed from the keratin by the action of alkali. See Gortner (1910).

0.1713 gm. gave 14.2 cc. N at 27.5° and 768 mm.

0.2243 gm. gave 18.5 cc. N at 25° and 767 mm.

0.1625 gm. gave 0.3143 gm. CO<sub>2</sub> and 0.0852 gm. H<sub>2</sub>O.

0.1584 gm. gave 0.3050 gm. CO<sub>2</sub> and 0.0804 gm. H<sub>2</sub>O.

*Found:*

Ash	=	0.84 and 0.84 per cent.	Average	=	0.84 per cent.
Ash-free sulphur	=	1.05 and 1.07 per cent.	Average	=	1.06 per cent.
Ash-free nitrogen	=	9.30 and 9.44 per cent.	Average	=	9.37 per cent.
Ash-free hydrogen	=	5.92 and 5.73 per cent.	Average	=	5.82 per cent.
Ash-free carbon	=	53.20 and 52.94 per cent.	Average	=	53.07 per cent.
Oxygen (by diff.)				=	30.68 per cent.

10. *Extraction with a 5 per cent Solution of Sodium Hydrate.*

Three hundred grams of black wool were boiled for four hours with 1 liter of 5 per cent solution of sodium hydrate. The solution was filtered through cheese cloth and the pigment purified as above. The same general description holds true for this product. Yield, 10.95 grams, or 3.65 per cent of the air-dry wool.<sup>1</sup>

ANALYSIS:

0.2083 gm. gave 0.0021 gm. ash.

0.2278 gm. gave 0.0021 gm. ash.

0.5328 gm. gave 0.0406 gm. BaSO<sub>4</sub> = 0.00558 gm. S.

0.5084 gm. gave 0.0392 gm. BaSO<sub>4</sub> = 0.00538 gm. S.

0.1490 gm. gave 12.0 cc. N at 22.5° and 768 mm.

0.2733 gm. gave 22.0 cc. N at 23.5° and 768 mm.

0.1615 gm. gave 0.3117 gm. CO<sub>2</sub> and 0.0815 gm. H<sub>2</sub>O.

0.1419 gm. gave 0.2767 gm. CO<sub>2</sub> and 0.0724 gm. H<sub>2</sub>O.

*Found:*

Ash	=	1.00 and 0.82 per cent.	Average	=	0.96 per cent.
Ash-free sulphur	=	1.05 and 1.06 per cent.	Average	=	1.05 per cent.
Ash-free nitrogen	=	9.21 and 9.23 per cent.	Average	=	9.22 per cent.
Ash-free hydrogen	=	5.71 and 5.71 per cent.	Average	=	5.71 per cent.
Ash-free carbon	=	53.13 and 53.18 per cent.	Average	=	53.16 per cent.
Oxygen (by diff.)				=	30.86 per cent.

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<sup>1</sup> See footnote to Tables II and III.

11. *Extraction with a 6 per cent Solution of Sodium Hydrate.*

Four hundred grams of air-dry black wool were boiled for four hours with 2 liters of 6 per cent sodium hydrate solution and the pigment isolated and purified as above. The same general properties were observed. Yield, 7.92 grams, or 1.98 per cent of the air-dry wool.

## ANALYSIS:

Unfortunately the container was broken before the analysis was undertaken and, inasmuch as it was feared that broken glass might contaminate the product, no analytical data were secured.

12. *Extraction with a 10 per cent Solution of Sodium Hydrate.*

Three hundred grams of black wool were boiled for four hours with 1 liter of 10 per cent solution of caustic soda, and the pigment isolated and purified as above. The same general properties were found. Yield, 7.35 grams, or 2.45 per cent of the air-dry wool.<sup>1</sup>

## ANALYSIS:

0.1594 gm. gave 0.0012 gm. ash.

0.1325 gm. gave 0.0010 gm. ash.

0.5050 gm. gave 0.0411 gm.  $\text{BaSO}_4$  = 0.00605 gm. S.

0.3550 gm. gave 0.0332 gm.  $\text{BaSO}_4$  = 0.00456 gm. S.

0.1892 gm. gave 11.6 cc. N at 23.5° and 765 mm.

0.1690 gm. gave 10.4 cc. N at 21° and 764 mm.

0.1594 gm. gave 0.3254 gm.  $\text{CO}_2$  and 0.0676 gm.  $\text{H}_2\text{O}$ .

0.1454 gm. gave 0.2960 gm.  $\text{CO}_2$  and 0.0640 gm.  $\text{H}_2\text{O}$ .

*Found:*

Ash	=	0.75 and 0.75 per cent.	Average	=	0.75 per cent.
Ash-free sulphur	=	1.20 and 1.29 per cent.	Average	=	1.24 per cent.
Ash-free nitrogen	=	6.99 and 7.08 per cent.	Average	=	7.03 per cent.
Ash-free hydrogen	=	4.79 and 4.97 per cent.	Average	=	4.88 per cent.
Ash-free carbon	=	56.09 and 55.94 per cent.	Average	=	56.01 per cent.
Oxygen (by diff.)				=	30.84 per cent.

13. *Extraction with a 20 per cent Solution of Sodium Hydrate.*

Three hundred grams of black wool were boiled for four hours with 1 liter of 20 per cent solution of sodium hydrate. The resulting solution was filtered through cheese cloth and poured into 9 liters of water containing

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<sup>1</sup> Through an error, this has elsewhere (Gortner, 1910) been reported as 1.83 per cent, the weight of wool being given as 400 grams instead of 300 grams.



600 cc. of concentrated hydrochloric acid. The precipitated pigment was purified as above and was found to have the same general properties. Yield, 5.42 grams, or 1.80 per cent.

**ANALYSIS:**

0.1203 gm. gave 0.0016 gm. ash.

0.1167 gm. gave 0.0015 gm. ash.

0.4080 gm. gave 0.0358 gm.  $\text{BaSO}_4$  = 0.0049 gm. S.

0.3202 gm. gave 0.0304 gm.  $\text{BaSO}_4$  = 0.0042 gm. S.

0.1257 gm. gave 6.8 cc. N at  $22.5^\circ$  and 762 mm.

0.1500 gm. gave 8.2 cc. N at  $26^\circ$  and 764 mm.

0.1589 gm. gave 0.3257 gm.  $\text{CO}_2$  and 0.0604 gm.  $\text{H}_2\text{O}$ .

0.1585 gm. gave 0.3237 gm.  $\text{CO}_2$  and 0.0594 gm.  $\text{H}_2\text{O}$ .

**Found:**

Ash	=	1.33 and 1.28 per cent.	Average	=	1.30 per cent.
Ash-free sulphur	=	1.22 and 1.32 per cent.	Average	=	1.27 per cent.
Ash-free nitrogen	=	6.20 and 6.18 per cent.	Average	=	6.19 per cent.
Ash-free hydrogen	=	4.31 and 4.25 per cent.	Average	=	4.28 per cent.
Ash-free carbon	=	56.60 and 56.44 per cent.	Average	=	56.52 per cent.
Oxygen (by diff.)				=	31.74 per cent.

**14. Extraction with a 30 per cent Solution of Sodium Hydrate.**

Three hundred grams of black wool were boiled with 1 liter of 30 per cent caustic soda solution for four hours, filtered through cheese cloth and poured into 9 liters of water containing 1000 cc. of concentrated hydrochloric acid. The precipitated pigment was purified as usual and was found to have the same general properties as the other preparations. It was found that, with increasing strengths of alkali, the alcohol-soluble portion was rapidly increased and, for that reason, the extraction with alcohol occupied considerable time. The alcohol-soluble product is a brown powder when precipitated by the addition of a large excess of ether, but was not further investigated. Yield of pigment, 5.17 grams, or 1.72 per cent of the air-dry wool.

**ANALYSIS:**

0.1067 gm. gave 0.0008 gm. ash.

0.1589 gm. gave 0.0012 gm. ash.

0.4325 gm. gave 0.0462 gm.  $\text{BaSO}_4$  = 0.00634 gm. S.

0.4362 gm. gave 0.0457 gm.  $\text{BaSO}_4$  = 0.00628 gm. S.

0.1510 gm. gave 6.8 cc. N at 21° and 772 mm.

0.1544 gm. gave 6.7 cc. N at 23° and 773 mm.

0.1634 gm. gave 0.3369 gm. CO<sub>2</sub> and 0.0620 gm. H<sub>2</sub>O.

0.1637 gm. gave 0.3387 gm. CO<sub>2</sub> and 0.0627 gm. H<sub>2</sub>O.

**Found:**

Ash	=	0.75 and 0.75 per cent.	Average =	0.75 per cent.
Ash-free sulphur	=	1.48 and 1.45 per cent.	Average =	1.46 per cent.
Ash-free nitrogen	=	5.24 and 5.01 per cent.	Average =	5.12 per cent.
Ash-free hydrogen	=	4.28 and 4.32 per cent.	Average =	4.30 per cent.
Ash-free carbon	=	56.59 and 56.84 per cent.	Average =	56.71 per cent.
Oxygen (by diff.)				= 32.41 per cent.

**15. Extraction with a 50 per cent Solution of Sodium Hydrate.**

Three hundred grams of black wool were heated to above 100° for four hours<sup>1</sup> with 1 liter of a 50 per cent solution of sodium hydrate. The solution was filtered through cheese cloth and poured into 9 liters of water containing 1750 cc. of concentrated hydrochloric acid. The pigment was purified as usual and possessed the same characteristics. Yield, 4.76 grams or 1.58 per cent of the air dry wool.

**ANALYSIS:**

0.1072 gm. gave 0.0010 gm. ash.

0.1279 gm. gave 0.0012 gm. ash.

0.4550 gm. gave 0.0404 gm. BaSO<sub>4</sub> = 0.00555 gm. S.

0.4094 gm. gave 0.0389 gm. BaSO<sub>4</sub> = 0.00534 gm. S.

0.2192 gm. gave 17.0 cc. N at 18° and 764 mm.

0.1370 gm. gave 10.7 cc. N at 23.5° and 762 mm.

0.1438 gm. gave 11.2 cc. N at 21° and 758 mm.

0.1500 gm. gave 0.3112 gm. CO<sub>2</sub> and 0.0516 gm. H<sub>2</sub>O.

0.1562 gm. gave 0.3235 gm. CO<sub>2</sub> and 0.0526 gm. H<sub>2</sub>O.

**Found:**

Ash	=	0.93 and 0.94 per cent.	Average =	0.93 per cent.
Ash-free sulphur	=	1.23 and 1.31 per cent.	Average =	1.27 per cent.
Ash-free nitrogen	=	9.11, 8.88 & 8.92 per cent.	Aver. =	8.97 per cent.
Ash-free hydrogen	=	3.89 and 3.80 per cent.	Average =	3.84 per cent.
Ash-free carbon	=	57.11 and 56.99 per cent.	Average =	57.06 per cent.
Oxygen (by diff.)				= 28.86 per cent.

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<sup>1</sup> Boiling actively was impossible in that the frothing of the solution threatened to cause the loss of a part of the material.

Much difficulty was experienced in the Dumas nitrogen determination of this preparation, inasmuch as the combustion progressed extremely slowly, even after the substance had been subjected to an intense red heat. The first rush of nitrogen was normal and corresponded to about 6 per cent of the original melanin. The remainder, however, was evolved extremely slowly. No difficulty in this respect was experienced with any other preparation.

The high nitrogen is, perhaps, due to the fact that the solution did not actually boil, and also the possibility presents itself that, under the influence of the strong caustic, some ammonia may have gone into combination with the decomposing pigment, or, that a portion of the pigment, containing no nitrogen, may have been split off thereby raising the nitrogen content in the remaining pigment. The latter appears the most probable explanation.

16. *Extraction with 25 per cent Sulphuric Acid.* Inasmuch as the hydrolysis of protein by mineral acids usually produces humic products, often in very large amount,<sup>1</sup> it was thought advisable to prepare the melanin from black wool using the method usually employed for the determination of tyrosin.

Three hundred grams of black wool were boiled for twenty-four hours with 2 liters of 25 per cent sulphuric acid. The jet black solution was filtered from the insoluble residue<sup>2</sup> and the black residue thoroughly washed. The purification of this product was carried out as in all the other preparations. Yield, 6.16 grams, or 2.05 per cent of the air dry wool.

#### ANALYSIS:

0.1346 gm. gave 0.0030 gm. ash.

0.1128 gm. gave 0.0026 gm. ash.

0.4196 gm. gave 0.0539 gm.  $\text{BaSO}_4$  = 0.0075 gm. S.

0.3552 gm. gave 0.0422 gm.  $\text{BaSO}_4$  = 0.0058 gm. S.

0.1968 gm. gave 9.5 cc. N at 24° and 765 mm.

0.2416 gm. gave 11.6 cc. N at 26° and 764 mm.

0.1644 gm. gave 0.3404 gm.  $\text{CO}_2$  and 0.0645 gm.  $\text{H}_2\text{O}$ .

0.1499 gm. gave 0.3108 gm.  $\text{CO}_2$  and 0.0564 gm.  $\text{H}_2\text{O}$ .

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<sup>1</sup> Abderhalden and Rilliet (1908-09) obtained 35.5 grams of humin by the hydrolysis of 220 grams of silk fibroin.

<sup>2</sup> From 300 grams of white wool by the same method the author obtained 0.8 gram of a brownish-black body which was not further investigated.

Found:

Ash	= 2.23 and 2.30 per cent.	Average = 2.26
Ash-free sulphur	= 1.83 and 1.67 per cent.	Average = 1.75
Ash-free nitrogen	= 5.52 and 5.48 per cent.	Average = 5.50
Ash-free hydrogen	= 4.49 and 4.31 per cent.	Average = 4.40
Ash-free carbon	= 57.77 and 57.85 per cent.	Average = 57.81
Oxygen (by diff.)		= 30.51

17. *An Analysis of the Melanin Ash:* In order to determine whether or not the ash had any significance an approximate analysis was undertaken.

A mixed sample of melanin weighing 3.1367 grams was placed in a platinum crucible giving 0.0321 grams of ash. The ash was treated in the platinum crucible with 6 cc. of concentrated hydrochloric acid and evaporated to dryness on a water bath, then dissolved in 2 cc. of concentrated hydrochloric acid, evaporated to dryness and baked at 110°–120°. The contents were then warmed in 10 cc. of dilute hydrochloric acid, filtered and the filtrate free of chlorides and ignited giving a pure white product.

The hydrochloric acid solution was analyzed according to Fildes's (1903) method for the analysis of soil solutions. The results obtained are shown in Table I.

TABLE I.  
*Analysis of Melanin Ash.*

PRODUCT	WEIGHT	PER CENT MELANIN ASH	PER CENT OF TOTAL
SiO <sub>2</sub> .....	0.0033	10.28	0.01
Fe <sub>2</sub> O <sub>3</sub> .....	0.00175	5.16	0.005
BaSO <sub>4</sub> .....	0.0248	.....	.....
SO <sub>3</sub> (calc.) .....	0.0085	25.83	0.025
CaO.....	0.0030	9.11	0.009
Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub> .....	0.0030	.....	.....
MgO .....	.....	3.40	0.003
NaCl + KCl.....	0.0159	.....	.....
K <sub>2</sub> PO <sub>4</sub> .....	0.0079	.....	.....
K <sub>2</sub> O.....	.....	4.45	0.004
Na <sub>2</sub> O.....	.....	20.66	0.02
Total.....		78.89	0.078
Not determined CO <sub>2</sub> , P <sub>2</sub> O <sub>5</sub> , Al <sub>2</sub> O <sub>3</sub> , etc....		21.11	0.021
		100.00	1.00

On account of the small amount of material available a great amount of accuracy is not claimed for these data. The figures do serve to show, however, that the ash is not a part of the melanin molecule, but is merely present as an impurity. The high sulphur content doubtless comes from the oxidation of the sulphur in the melanin molecule, while the presence of such a large portion of sodium probably originated in the alkali used for extraction. The silica probably comes from the glassware, although "Jena" glass was used wherever possible in order to eliminate ash from this source as completely as possible.

III. THE RESULTS.

18. *A Summary of the Experimental Data:* For ease of both expression and reference it has been thought advisable to express the experimental summary in the form of tables:

TABLE II.

*Showing the Percentage Yields of Ash-free Melanin Obtained by Extraction with Increasing Strengths of Alkali.*

	0.2% NaOH	1% NaOH	2.5% NaOH	5% NaOH	6% NaOH	10% NaOH	20% NaOH	30% NaOH	50% NaOH	25% H <sub>2</sub> SO <sub>4</sub>
Acid-soluble pigment.....	8.10									
Acid-insoluble pigment.....	1.36	3.26	2.95	3.62*	1.95	2.43	1.78	1.71	1.56	2.00

\* Unfortunately this determination was made on wool shorn a year later than the other materials. The figures therefore, are not strictly comparable. The same will also apply to the analytical data.

TABLE III.

*Showing the Analytical Data Obtained by a Study of the Various Pigments Isolated by the Different Methods.*

METHOD	C	H	N	S	(BY DIFF) O	RATIOS* S = 1
0.2 per cent NaOH soluble in acid.	52.60	7.28	13.52	1.33	25.25	C <sub>106</sub> H <sub>173</sub> N <sub>23</sub> SO <sub>28</sub>
0.2 per cent insol. in acid.	53.44	5.81	10.44	1.16	29.15	C <sub>124</sub> H <sub>160</sub> N <sub>21</sub> SO <sub>31</sub>
0.2 per cent NaOH from soluble pig- ment insol. in acid.	54.37	5.54	9.48	1.23	29.38	C <sub>120</sub> H <sub>146</sub> N <sub>19</sub> SO <sub>30</sub>
1 per cent NaOH	52.20	6.62	10.34	1.06	29.78	C <sub>122</sub> H <sub>150</sub> N <sub>22</sub> SO <sub>34</sub>
2.5 per cent NaOH	53.07	5.82	9.37	1.06	30.68	C <sub>127</sub> H <sub>175</sub> N <sub>20</sub> SO <sub>33</sub>
5 per cent NaOH	53.16	5.71	9.22	1.05	30.86	C <sub>124</sub> H <sub>172</sub> N <sub>20</sub> SO <sub>33</sub>
10 per cent NaOH	56.01	4.88	7.03	1.24	30.84	C <sub>120</sub> H <sub>125</sub> N <sub>12</sub> SO <sub>40</sub>
20 per cent NaOH	56.52	4.28	6.19	1.27	31.74	C <sub>118</sub> H <sub>106</sub> N <sub>11</sub> SO <sub>40</sub>
30 per cent NaOH	56.71	4.30	5.12	1.46	32.41	C <sub>102.5</sub> H <sub>92.5</sub> N <sub>9</sub> SO <sub>40</sub>
50 per cent NaOH	57.06	3.84	8.98	1.27	28.85	C <sub>119</sub> H <sub>95</sub> N <sub>16</sub> SO <sub>45</sub>
25 per cent H <sub>2</sub> SO <sub>4</sub>	57.81	4.40	5.50	1.75	30.52	C <sub>88</sub> H <sub>80</sub> N <sub>7</sub> SO <sub>28</sub>

\*The author wishes it distinctly understood that the ratios are not given as formulas of compounds isolated. Perhaps the first ratio does represent a definite chemical compound. The others do not. They are only given as a guide in understanding the decomposing effect of caustic alkali.

19. *A Discussion of the Experimental Data:* From the above tables it will readily be seen that caustic alkali, except in very small concentrations, is not without effect upon the coloring matter of black wool, for a slight increase in the concentration causes a loss of more than 50 per cent of the pigment molecule.

The percentage of sulphur remains remarkably constant considering the drastic treatment. The rise in sulphur content in the product formed by the hydrolysis with sulphuric acid being probably due to the presence of some sulphuric acid addition product or else to the presence of sulphates.

The nitrogen content is very sensitive to the action of alkali, falling steadily from 13.52 per cent to 5.12 per cent with 30 per cent alkali. I am unable to offer any explanations other than those given on page 357 for the abnormal behavior of the 50 per cent alkali. Enough consistent data have already been secured,

however, to show that nitrogen is very readily lost when melanin is treated with either strong alkali or acid.

Although other writers have to some extent recognized the danger of loss of nitrogen and sulphur, I have been unable to find any reference to the possibility of a loss of hydrogen content. Here we find a steady and rapid fall from a maximum of 7.28 per cent to 3.84 per cent with 50 per cent alkali, the fall being much in excess of the amount which would be lost were the hydrogen split off wholly as ammonia.

Corresponding to the loss of hydrogen and nitrogen we should look for a gain in either oxygen or carbon or in both. The latter we find to be the case, carbon rising from 52.60 per cent to 57.06 per cent with 50 per cent alkali and to 57.81 per cent by the acid hydrolysis, and oxygen rising at the same time from 25.25 per cent to 32.41 per cent with the 30 per cent solution of caustic soda, the 50 per cent figures being affected by the abnormal nitrogen determination.

Spiegler (1903), in his study of the pigment from black wool, reports the following average analysis, the figures being calculated for ash-free pigment: C, 50.95 per cent; H, 6.14 per cent; N, 10.28 per cent; S, 2.91 per cent; O, 29.72 per cent. This analysis shows less carbon than in any of my preparations, a hydrogen content equal to an alkali concentration of between 1 and 2.5 per cent on Table III, a nitrogen content about equivalent to mine found by 1 per cent alkali, and a sulphur content which is double that found for any of my products which were obtained by the action of caustic soda.

The low carbon content of Spiegler's product, it seems very probable, is due to the formation of carbonates or carbides during the process of combustion. Spiegler makes no mention of any attempt to determine whether or not such products were present, and inasmuch as he reports *an ash content of 10.85 per cent* it is very improbable that he obtained all of the carbon present in the melanin. The nitrogen and hydrogen data are also, probably, affected to some extent by the presence of ammonium salts in the ash, inasmuch as he used ammonia to dissolve his product during the purification process. The abnormal sulphur determination is, doubtless, due to the presence of sulphates or sulphonic acids, or similar combination or addition products, formed when he dissolved his pigment in

concentrated sulphuric acid. This view is upheld by the fact that I found the sulphur content to be greatly increased when the pigment was isolated by hydrolysis with 25 per cent sulphuric acid.

#### IV. SUMMARY.

20. *Résumé:* 1. The effect of varying strengths of alkali upon the composition of melanin of black wool has been studied.

2. This study shows that alkali, except in very small concentrations, readily destroys the greater portion of the melanin molecule.

3. By the use of alkali in strengths not exceeding a 0.2 per cent solution a melanin has been isolated which is readily soluble in acetic or formic acids, and in mineral acids of strengths not exceeding tenth-normal.

4. This melanin is apparently of constant composition and contains no ash.

5. The nitrogen content of this melanin is readily attacked by the action of alkali falling from 13.52 per cent to 5.12 per cent when treated with 30 per cent caustic soda.

6. The sulphur content remains practically constant when treated with alkali.

7. The hydrogen content falls rapidly under the influence of alkali, dropping from a maximum of 7.28 per cent to a minimum of 3.84 per cent with 50 per cent caustic soda.

8. Both the carbon and oxygen percentages are increased as the molecule is broken down under the influence of increasing strengths of sodium hydrate.

9. The study of the acid-soluble melanin is being continued as are also studies on the acid-soluble melanins of black and brown horse hair, human hair, etc.

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# AN IMPROVEMENT OF THE FOLIN METHOD FOR THE DETERMINATION OF URINARY AMMONIA NITROGEN.<sup>1</sup>

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## I. INTRODUCTION.

### *The Folin Method is Inadequate for the Quantitative Liberation of Ammonia from Triple Phosphate.*

In the fall of 1907, during the progress of a metabolism research on "the influence of magnesium sulphate on metabolism,"<sup>2</sup> certain anomalous results were obtained in our quantitative determination of the urinary ammonia. In the earlier periods of that research the ammonia had been determined in duplicate by the Folin method, in the daily urines for nearly six weeks, with thoroughly concordant results. But throughout a metabolism period during which magnesium sulphate was injected subcutaneously once every twenty-four hours, the titration results in duplicate (at the conclusion of the Folin process as applied to the daily urines) were strikingly discordant, the disagreements amounting to from 1 to 2 cc. of  $\frac{N}{5}$  potassium hydroxide solution per 25 cc. of urine.

Our inability to obtain, in duplicate determinations, concordant results for the quantities of ammonia in the urines collected during the period of the magnesium sulphate treatment, or to explain

<sup>1</sup> Preliminary references to the experiments described in this paper were published in the *Proc. of the Soc. for Exp. Biol. and Med.*, vi, p. 127, 1909, and in the *Proceedings of the American Society of Biological Chemists*, i, p. 1, 1910; also this *Journal*, vii, p. lviii, 1910.

<sup>2</sup> Steel: this *Journal*, v, p. 85, 1908.

these disagreements by any probable fault of technique, led us to make two general suppositions regarding the cause of the analytical discrepancies:

1. That the injected magnesium was eliminated into the urine in question in relatively large quantities as ammonio-magnesium phosphate, which separated, in part at least, in typically crystalline masses.

2. That the crystalline ammonio-magnesium phosphate thus deposited was not thoroughly decomposed by sodium carbonate as employed in the Folin process, whereby ammonia remained, in variable amounts, in its solid form as triple phosphate in the urine under investigation.

General examination of the urines that gave the anomalous results for ammonia content showed at a glance that our first supposition was correct—triple phosphate had crystallized in abundance. However, in separating portions of the urines for analysis care had always been taken to isolate fractions of the thoroughly shaken and evenly mixed daily samples. Consequently, there was no reason to believe that any of the above-mentioned anomalous results of the ammonia determinations had been due to transference of unequal amounts of the deposited ammonio-magnesium phosphate in the fractions of the urine taken. We therefore proceeded to test very carefully, and in many trials, the validity of the second supposition stated above. Our investigation proved unmistakably the correctness of the conclusion already drawn, viz. that the Folin method fails, in the case of triple phosphate, to give accurate results for ammonia content, the error amounting to much as 53.3 per cent.<sup>1</sup>

## II. ANALYTICAL.

### *The Inadequacy of Other Methods.*

We next endeavored to find some other method for the determination of ammonia that would liberate all the ammonia from crystalline ammonio-magnesium phosphate and at the same time give satisfactory results in urinary analysis. Consequently,

<sup>1</sup> Steel and Gies: this *Journal*, v, p. 71, 1908.

every other published method that involved a different reagent, but none fulfilled both conditions. The Shaffer method<sup>1</sup> gave the best results, but it likewise failed to liberate all the ammonia from triple phosphate. The Ronchèse method,<sup>2</sup> which is based on the fact that formaldehyde reacts with ammonium chloride and produces from them hexa-methylenetetramine,  $\text{4Cl} + 6\text{CH}_2\text{O} + 4\text{NaOH} = \text{N}_4(\text{CH}_2)_6 + 10\text{H}_2\text{O} + 4\text{NaCl}$ , failed to liberate all the ammonia from triple phosphate, but on normal urines it always gave much higher results for ammonia than the Folin or other methods. On normal urines this excess amounted to as much as 19 per cent (compared with data obtained by the Folin method). Results in this connection are given in Table 1.

TABLE I.

*Comparison Between Some Results Obtained with the Ronchèse and Folin Methods for the determination of Urinary Ammonia Nitrogen.*

MILLIGRAMS OF AMMONIA NITROGEN IN 100 CC. OF URINE.			PERCENTAGE EXCESS OF THE RONCHÈSE AMOUNT OVER THE FOLIN QUANTITY.
Urine.	Folin Method.	Ronchèse Method.	
Urine I	1 57.91	68.21	17.78
	2 57.91	68.16	17.69
	3 57.35	67.51	17.71
Urine II	1 66.84	74.40	11.31
	2 66.70	74.56	11.79
Urine III	1 36.85	43.00	16.68
	2 36.70	42.84	16.73
Urine IV	1 82.24	89.64	8.99
	2 82.24	89.88	9.28
Urine V	1 94.80	112.48	18.64
	2 94.45	112.48	19.00

As shown in Table I will show that while the Ronchèse method indicated a greater ammonia content than the Folin method, the increase was quite uniform for a given urine. It seemed probable, therefore, that the Ronchèse method liberated from the urine some definite amount of some substance or substances other than ammonia. In order to ascertain, if possible, what caused this

<sup>1</sup>Shaffer: *Amer. Journ. of Physiol.*, viii, p. 330, 1902-3.  
<sup>2</sup>Ronchèse: *Journ. de pharm. et de chimie*, xxv, p. 611, 1907.

difference, we added weighed amounts of non-ammoniacal substances to different 10 cc. fractions of a sample of normal urine and compared the ammonia results with those obtained from the original urine. The data in table II suggest that the plus error was due, in part at any rate, to amino acids, a conclusion in harmony with Sørensen's<sup>1</sup> observations.

TABLE II.

*Results Showing the Effects of Equal Weights of Various Non-ammoniacal Substances Added to 10 cc. Samples of a Given Normal Urine on the Amount of Apparent Ammonia Recovered by the Ronchèse Method.*

NUMBER OF DETERMINATIONS.	ADDED SUBSTANCE.		QUANTITY OF AMMONIA NITROGEN PER 10 CC. OF URINE AS INDICATED BY THE RONCHÈSE METHOD.
	Name.	Amount.	
		gram.	milligrams.
4	Control Test	None	6.86
2	Hippuric acid	0.1	6.79
3	Urea	0.1	6.86
2	Uric acid	0.1	6.89
2	Leucin*	0.1	7.28
2	Tyrosin*	0.1	8.82
2	Guanin*	0.1	8.96
2	Taurin*	0.1	12.74
2	Glycocoll*	0.1	24.50

\* Other portions of these preparations were later used in comparable determinations by the Folin and our modified methods (Table VII), and with those methods no extra ammonia was indicated, thereby showing that these very high results were not due to impurities.

Other modifications of the formaldehyde method have been reported, notably that of Malfatti.<sup>2</sup> The difference between his method and that of Ronchèse consists in the use of less formaldehyde. We found that this difference offers no advantage. In fact, the results with the Malfatti method are not nearly so accurate as those of the Ronchèse method. Ronchèse<sup>3</sup> showed that an excess of formaldehyde helped to make the results most accurate. Matheson<sup>4</sup> claims that the accuracy of the Malfatti method is

<sup>1</sup> Sørensen: *Biochem. Zeitschr.*, vii, p. 47, 1908.  
<sup>2</sup> Malfatti: *Zeitschr. f. anal. Chemie*, xvlii, p. 273, 1908.  
<sup>3</sup> Ronchèse: *loc. cit.*  
<sup>4</sup> Matheson: *Brit. Med. Journ.*, 1909, p. 715.

ed by adding 15 grams of potassium oxalate and shaking  
utes before titrating. The end point, he claims, is sharp-  
r the precipitation of the calcium salts and also because  
nution of the influence of the ammonium salts. Both  
and ammonium salts have a disturbing influence on the  
nt of the first titration in the Malfatti method. Matheson's  
on that the end point is sharpened we found to be correct,  
modification is of no practical value because it always  
increase the plus error several per cent. His figures show  
d our own accord with his. There was always a *small*  
in the ammonia figure when potassium oxalate was added.  
n<sup>1</sup> has recently published a paper on the clinical estimation  
onia in urine by the formalin method. In this paper the  
discusses the original Ronchèse method, also the various  
d modifications of the method. The modification which  
ders to be of greatest value is the one suggested by Mathe-  
t was referred to above (addition of potassium oxalate).  
other investigators, however, Brown obtained much higher  
for ammonia with the formalin method than with other  
d processes such as the Shaffer method. To reduce this  
or Brown clarified the urine with basic lead acetate, and  
ployed the Matheson modification of the Ronchèse method.  
claimed that this procedure reduced the plus error (com-  
ith the data obtained by the Shaffer method) from 20 per  
15 per cent down to an average of 6 per cent, a difference  
e considers to be of no clinical importance. The author  
explain how the addition of basic lead acetate reduced the  
or. In fact, he specifically stated that basic lead acetate  
ot precipitate amino-acids, which substances have been  
y other investigators to be the source of the error in ques-  
brown's modification is, therefore, of little real value.

#### *Improvement of the Folin Method.*

e none of the existing methods gave us accurate results  
nonia content in urines containing triple phosphate crys-

n: *Journ. Amer. Med. Assn.*, liii, p. 2071, 1909.

eson: *loc. cit.*

tals, we were obliged either to devise a new method, or else modify some accepted method to meet the requirement indicated. We naturally chose the latter course, and the Folin process, being the simplest and best method with normal urines, was the one selected.

We first attempted to find an alkali which would liberate all the ammonia from pure ammonio-magnesium phosphate crystals and from all true ammoniacal compounds, but which at the same time would not produce ammonia from any of the amino substances such as urea, that occur in urine.

Different amounts of sodium hydroxide, milk of lime, saturated baryta water and magnesium oxide were added to weighed amounts of triple phosphate in given volumes of water (25 cc.) and aëration was conducted as in the Folin process.

TABLE III.

*Data Showing the Proportions of Ammonia Liberated from Weighed Amounts of Triple Phosphate in 25 cc. of Water by Different Alkalies under Identical Conditions.*

NH <sub>4</sub> MgPO <sub>4</sub>	ALKALI USED TO LIBERATE THE AMMONIA FROM THE TRIPLE PHOSPHATE.		VOLUME OF STANDARD ACID SOLUTION REQUIRED FOR NEUTRALIZATION OF THE AMMONIA LIBERATED.		PROPORTION OF AMMONIA LIBERATED IN THE PROCESS
Weight.	Kind.	Quantity.	After Aëration for 4 Hours.	If All NH <sub>3</sub> Had Been Liberated.	
gram.		gram.	cc.	cc.	per cent.
0.5	Sodium hydroxide	0.5	10.25	10.3	None
0.5		0.5	10.30	10.3	
0.5		1	10.30	10.3	
0.5		1	10.35	10.3	
		c.c.			
0.5	Milk of lime	20	6.30	10.3	38.8
0.5		40	6.95	10.3	32.0
0.5		100	7.75	10.3	24.8
		c.c.			
0.5	Baryta water	20	4.40	10.3	57.2
0.5		40	4.85	10.3	52.9
0.5		100	5.10	10.3	50.5
		gram.			
0.5	Magnesium oxide	2	1.75	10.3	83.0
0.5		5	2.05	10.3	80.0
0.5		10	2.05	10.3	80.0



The data in Table III make it evident that milk of lime, baryta water and magnesium oxide, in the proportions employed, would not be strong enough to liberate all the ammonia from a urine containing a large proportion of ammonio-magnesium phosphate crystals, whereas sodium hydroxide, even in comparatively small amounts, would be very efficient in that direction. We next endeavored to ascertain whether such small proportions of sodium hydroxide would decompose any of the ordinary amino constituents of urine.

Few investigators seem to have tried caustic alkali in the development of their methods of ammonia liberation. Munk,<sup>1</sup> used sodium hydroxide in a modification of the Schlösing method. He found that caustic soda, when dissolved in urine, gradually evolved ammonia from non-ammoniacal material and therefore gave the results that were too high.

Fränkel<sup>2</sup> employed potassium hydroxide in a modification of the same method but found that in two hours all the ammonia was liberated and that no additional ammonia was formed when urea was added to the ammonia-free urine.

In the light of the above data we tried, on normal urines, the simple substitution of one gram of sodium hydroxide for the sodium carbonate in the Folin method. The result was not entirely satisfactory. There was usually a slight excess in the amount of ammonia thus obtained over the quantity that was recovered when sodium carbonate was used (see Table IV).

The small plus difference might be explained by assuming that the modified process is more complete in its liberating effect, but it seemed much more probable that the excess was due to a slight decomposition of urea by the caustic alkali. Folin, in the first description of his method, advised the use of sodium chloride in addition to sodium carbonate, to prevent such a decomposition, but later he abandoned the use of sodium chloride on the ground that it was unnecessary. In this laboratory we have found such to be the case. It was considered probable, however, that if it is desired to use caustic alkali satisfactorily instead of sodium carbonate as an improvement of the Folin method, a fairly large quantity of

<sup>1</sup> Munk: *Virchow's Archiv.*, lxxviii, p. 365, 1877.

<sup>2</sup> Fränkel: *Bull. soc. chim.*, xxv, p. 250, 1906.

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sodium chloride should accompany it, in order to reduce the dissociation of the alkali and thus prevent decomposition by it. Consequently, about 15 grams of sodium chloride were added to mixtures like those referred to in Table IV, and the tests frequently repeated.

TABLE IV.

*Data Showing the Effect on Normal Urine of the Substitution of Sodium Hydroxide for Sodium Carbonate in the Folin Method for the Determination of Urinary Ammonia.*

NORMAL URINE TAKEN.	ALKALI EMPLOYED.		VOLUME OF STANDARD ACID SOLUTION REQUIRED FOR NEUTRALIZATION OF THE LIB- ERATED $\text{NH}_3$ AFTER AERATION 3-4 HOURS.		DIFFERENCE IN FAVOR OF THE MODIFIED PROCESS.
	FOLIN METHOD.	MODIFIED PROCESS.			
Volume.	$\text{Na}_2\text{CO}_3$ .	$\text{NaOH}$	Folin Method.	Modified Process.	
cc.	grams.	gram.	cc.	cc.	cc.
25	3	1	9.10	9.20	0.10
25	3	1	7.30	7.30	
25	3	1	6.30	6.35	0.05
25	3	1	4.95	5.05	0.10
25	3	1	8.20	8.25	0.05
25	3	1	8.20	8.30	0.10
25	3	1	8.15	8.25	0.10

The results obtained, as shown in Tables V and VI, make it evident that the addition of sodium chloride was beneficial and that, with this modification, sodium hydroxide in the proportion indicated may be advantageously substituted for sodium carbonate in determining the urinary ammonia-nitrogen in normal urines.

Consequently, since small proportions of sodium hydroxide will eject all the ammonia from quantities of ammonio-magnesium phosphate far in excess of the amounts ever found in similar volumes of abnormal urine (Table III), our purpose appeared to be accomplished. Additional tests were made to remove all doubt, if possible.

In order to ascertain whether sodium hydroxide, in the proportions employed in the previous experiments and in the presence of a relatively large amount of sodium chloride, would produce ammonia from amino radicals, weighed amounts (0.1 gram) of

TABLE V.

*Data Showing the Effect on Normal Urines of Substituting Sodium Hydroxide Plus Sodium Chloride for Sodium Carbonate in the Folin Method for the Determination of Urinary Ammonia.*

Normal urine, 25 cc. Alkali: Sodium carbonate, 3 grams; or Sodium hydroxide, 1 gram, with sodium chloride, 15 grams (*instead of Sodium carbonate*). Period of aëration, 3-4 hours. Control test with 1 gram of NaOH and 15 grams of NaCl = 0.05 cc. of  $\frac{N}{3}$  acid.

NORMAL URINE TAKEN	FOLIN METHOD.		MODIFIED PROCESS.		VOLUME OF STANDARD ACID SOLUTION REQUIRED TO NEUTRALISE THE LIBERATED AMMONIA.		DIFFERENCE.	
	Vol- ume.	Na <sub>2</sub> CO <sub>3</sub>	NaOH	NaCl	Folin Method 3-4 hrs. Aëration.	Modified Pro- cess 3-4 hrs. Aëration.	In Favor of the Folin Method.	In Favor of the Modified Pro- cess.
cc.	grams.	gram.	grams.	cc.	cc.	cc.	cc.	cc.
25	3	1	15	7.70	7.65	0.05	....	
25	3	1	15	6.40	6.35	0.05	....	
25	3	1	15	5.90	6.00	....	0.10	
25	3	1	15	3.80	3.85	....	0.05	
25	3	1	15	5.90	5.95	....	0.05	
25	3	1	15	4.40	4.40	....	....	
25	3	1	15	4.15	4.15	....	....	
25	3	1	15	3.80	3.85	....	0.05	
25	3	1	15	4.80	4.75	0.05	....	
25	3	1	15	13.20	13.15	0.05	....	
25	3	1	15	8.05	8.00	0.05	....	
25	3	1	15	8.30	8.30	....	....	
25	3	1	15	6.55	6.55	....	....	
25	3	1	15	5.40	5.45	....	0.05	
25	3	1	15	5.40	5.35	0.05	....	
25	3	1	15	5.00	5.05	....	0.05	
25	3	1	15	4.95	4.95	....	....	
25	3	1	15	5.00	4.95	0.05	....	
25	3	1	15	4.15	4.15	....	....	
25	3	1	15	5.00	5.00	....	....	
25	3	1	15	4.30	4.35	....	0.05	
25	3	1	15	4.25	4.25	....	....	
25	3	1	15	5.30	5.30	....	....	
25	3	1	15	6.20	6.20	....	....	
25	3	1	15	6.20	6.20	....	....	
25	3	1	15	6.20	6.20	....	....	
25	3	1	15	5.35	5.30	0.05	....	
25	3	1	15	9.20	9.20	....	....	
25	3	1	15	6.15	6.15	....	....	
25	3	1	15	13.10	13.10	....	....	
25	3	1	15	10.10	10.10	....	....	
25	3	1	15	10.15	10.10	0.05	....	
25	3	1	15	9.00	9.00	....	....	
25	3	1	15	4.20	4.25	....	0.05	
25	3	1	15	4.20	4.20	....	....	
25	3	1	15	6.35	6.35	....	....	

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urea, uric acid, glycocoll, taurin, leucin, tyrosin, hippuric guanin, allantoin, and mixed creatin and creatinin<sup>1</sup> were added separately and collectively to 25 cc. fractions of normal urines after aëration in the usual manner, the amounts of liberated

TABLE VI.

*Data Showing the Effect on Normal Urines of Substituting Sodium Hydroxide Plus Sodium Chloride for Sodium Carbonate in the Folin Method for the Determination of Urinary Ammonia.*

Normal urine, 25 cc. Alkali: Sodium carbonate, 3 grams; or Sodium hydroxide, 0.5 gram, with sodium chloride, 15 grams (*instead of Sodium carbonate*). Period of aëration, 4 hours each (2). Control test: 0.5 gram of NaOH with 15 grams of NaCl = 0.05 cc.  $\frac{N}{5}$  acid.

NORMAL URINE TAKEN.	FOLIN METHOD.	MODIFIED PROCESS.		VOLUME OF STANDARD ACID SOLUTION REQUIRED TO NEUTRALIZE THE LIBERATED AMMONIA.						DIFFER
	Na <sub>2</sub> CO <sub>3</sub>	NaOH	NaCl	Folin Method.			Modified Process.			
				4 hrs. (1)	4 hrs. (2)	Total 8 hrs.	4 hrs. (1)	4 hrs. (2)	Total 8 hrs.	
Vol- ume.	Na <sub>2</sub> CO <sub>3</sub>	NaOH	NaCl	4 hrs. (1)	4 hrs. (2)	Total 8 hrs.	4 hrs. (1)	4 hrs. (2)	Total 8 hrs.	In Favor of the Folin Method.
cc.	grams.	gram.	grams.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
25	3	0.5	15	6.00	....	6.00	6.00	....	6.00	....
25	3	0.5	15	6.05	....	6.05	6.05	....	6.05	....
25	3	0.5	15	6.05	....	6.05	6.10	....	6.10	....
25	3	0.5	15	9.40	....	9.40	9.40	....	9.40	....
25	3	0.5	15	5.35	....	5.35	5.40	....	5.40	....
25	3	0.5	15	3.50	None	3.50	3.50	None	3.50	....
25	3	0.5	15	3.60	None	3.60	3.60	None	3.60	....
25	3	0.5	15	3.50	0.10	3.60	3.60	0.05	3.65	....
25	3	0.5	15	3.70	None	3.70	3.70	None	3.70	....
25	3	0.5	15	3.85	None	3.85	3.80	None	3.80	0.05
25	3	0.5	15	3.90	None	3.90	3.90	None	3.90	....
25	3	0.5	15	4.20	None	4.20	4.25	None	4.25	....
25	3	0.5	15	6.10	0.10	6.20	6.20	None	6.20	....
25	3	0.5	15	5.40	None	5.40	5.35	None	5.35	0.05
25	3	0.5	15	6.30	None	6.30	6.30	None	6.30	....
25	3	0.5	15	5.70	0.05	5.75	5.75	None	5.75	....
25	3	0.5	15	6.10	None	6.10	6.10	None	6.10	....
25	3	0.5	15	6.60	None	6.60	6.65	None	6.65	....

<sup>1</sup> The samples of "mixed creatin and creatinin" contained 51.45 per cent of creatinin and 48.55 per cent of creatin.

TABLE VII.

*Data Showing Effects of Added amounts of Various Urinary Nitrogenous Substances on the Quantities of Ammonia Obtained from Normal Urines by the Modified Method for the Determination of Urinary Ammonia.*

NORMAL URINE.		REAGENTS.		ADDED SUBSTANCE.		VOLUME OF STANDARD ACID SOLUTION REQUIRED TO NEUTRALISE THE LIBERATED $\text{NH}_3$ AFTER 4 HRS.
Sample No.	Volume Taken.	NaOH	NaCl	Name.	Amount	
	cc.	gram.	grams.		gram.	cc.
Sample I	1 25	0.5	15	Control test	None	5.20
	2 25	0.5	15	Leucin	0.1	5.20
	3 25	0.5	15	Tyrosin	0.1	5.15
	4 25	0.5	15	Glycocoll	0.1	5.20
	5 25	0.5	15	Urea	0.1	5.20
	6 25	0.5	15	Uric acid	0.1	5.25
	7 25	0.5	15	Taurin	0.1	5.20
	8 25	0.5	15	Hippuric acid	0.1	5.15
	9 25	0.5	15	Guanin	0.1	5.20
Sample II	1 25	0.5	15	Control test	None	6.10
	2 25	0.5	15	{ Creatin and creatinin }	0.1	6.10
	3 25	0.5	15	Allantoin	0.1	6.15
Sample III	1 25	0.5	15	Control test	None	5.80
	2 25	0.5	15	Control test	None	5.75
	3 25	0.5	15	{ Urea, uric acid, glycocoll, taurin, leucin, hippuric acid, tyrosin and guanin(each, 0.1) }	0.8	5.85
	4 25	0.5	15	{ Urea, uric acid, glycocoll, taurin, leucin, hippuric acid, tyrosin and guanin(each, 0.1) }	0.8	5.80
	5 25	0.5	15	{ Urea, uric acid, glycocoll, allantoin, and creatin and creatinin (each, 0.1) }	0.5	5.40
Sample V	1 25	0.5	15	Control test	None	5.40
	2 25	0.5	15	Control test	None	5.45
	3 25	0.5	15	{ Urea, uric acid, glycocoll, allantoin, and creatin and creatinin (each, 0.1) }	0.5	5.40

monia nitrogen were compared with those from the original urine. In no case, as shown by the data in Table VII, was any increase observed. These same substances were also added to 20 cc. of fractions of a standard solution of ammonium chloride instead of urine and the ammonia content determined. Here again no increase was obtained (Table VIII).

TABLE VIII.

*Data Showing Effects of Added Amounts of Various Urinary Nitrogenous Substances on the Quantities of Ammonia Obtained from a Given Solution of Ammonium Chloride by the Modified Method for the Determination of Urinary Ammonia.*

VOLUME OF NH <sub>4</sub> Cl SOLUTION.	REAGENTS.		ADDED SUBSTANCE.		VOLUME OF STANDARD ACID SOLUTION REQUIRED TO NEUTRALIZE THE LIBERATED NH <sub>3</sub> AERATION: 4 H
	NaOH	NaCl	Name.	Amount.	
cc.	gram.	grams.		gram.	cc.
20	0.5	15	Control test	None	9.80
20	0.5	15	Control test	None	9.85
20	0.5	15	Urea	0.5	9.80
20	0.5	15	Urea	0.5	9.85
20	0.5	15	Glycocoll	0.5	9.80
20	0.5	15	Glycocoll	0.5	9.80
20	0.5	15	Uric acid	0.5	9.75
20	0.5	15	Leucin	0.5	9.80
20	0.5	15	Tyrosin	0.5	9.85
20	0.5	15	Hippuric acid	0.5	9.80
20	0.5	15	Taurin	0.5	9.75
20	0.5	15	Guanin	0.5	9.80
20	0.5	15	Allantoin	0.5	9.80
20	0.5	15	Creatin and creatinin	0.5	9.85
20	0.5	15	{ Urea, glycocoll, uric acid, leucin, tyrosin, hippuric acid, taurin and guanin (each, 0.1) }	0.8	9.85
20	0.5	15	{ Urea, glycocoll, uric acid, leucin, tyrosin, hippuric acid, taurin, guan- in, allantoin, and creatin and crea- tinin (each, 0.1) }	1.0	9.80

These results show conclusively that the ordinary organic constituents of urine are not decomposed by the stated proportions of sodium hydroxide, in the presence of a physical excess of sodium chloride and under conditions favorable for the liberation of all the inorganic ammonia in normal urine.

As a final test of the efficiency of the modified method, the ammonia in fractions of a given sample of normal urine was separated by the Folin method and also by the modified method. To equal portions of the same specimen of urine, 0.5 gram samples of ammonio-magnesium phosphate crystals were added and the total ammonia content again determined by both methods. The ammonia in 0.5 gram portions of the triple phosphate was also separately obtained by each method. The results are given in Table IX.

TABLE IX.

*Data Showing the Effects of Added Triple Phosphate on the Amount of Ammonia Recovered from a Normal Urine by the Folin Method (3 grams of  $\text{Na}_2\text{CO}_3$ ) and by the Modified Method (1 gram  $\text{NaOH}$  + 15 grams  $\text{NaCl}$ ).*

NUMBER OF DETERMINATIONS.	MATERIAL ANALYZED.	METHOD USED.	VOLUME OF STANDARD ACID SOLUTION REQUIRED TO NEUTRALIZE THE LIBERATED AMMONIA.				
			After Aëration.			If all $\text{NH}_3$ had been liberated.	Ammonia lost
			2 hrs. (1)	4 hrs. (2)	Total 6 hrs.		
			cc.	cc.	cc.	cc.	%
2	Normal urine (25 cc.)	Folin	6.40	0.80	7.20	7.20	None
2	Normal urine (25 cc.)	Modified	6.55	0.65	7.20	7.20	None
2	25 cc. of water + 0.5 gram of $\text{NH}_4\text{MgPO}_4$	Folin	5.55	1.00	6.55	10.3	36.4
2	25 cc. of water + 0.5 gram of $\text{NH}_4\text{MgPO}_4$	Modified	9.70	0.60	10.30	10.3	None
2	25 cc. of water + 0.5 gram of $\text{NH}_4\text{MgPO}_4$	Kjeldahl			10.30	10.3	None
2	25 cc. of urine + 0.5 gram of $\text{NH}_4\text{MgPO}_4$	Folin	9.60	3.55	13.15	17.5	24.9
2	25 cc. of urine + 0.5 gram of $\text{NH}_4\text{MgPO}_4$	Modified	16.35	1.20	17.55	17.5	None

The results of the final test (Table IX) completely confirmed conclusion already drawn, to the effect that the Folin method fails, in the case of urines containing crystals of triple phosphate give perfectly accurate results for ammonia content. These results also still further emphasize our statement that sodium hydroxide in amounts varying from 0.5 to 1 gram plus about 15 gram sodium chloride, may be substituted for the sodium carbonate as prescribed in the Folin method.

### III. GENERAL CONCLUSION.

The data here presented prove conclusively that the modified method will eject all the ammonia from crystalline ammonio-magnesium phosphate in urine without producing ammonia from amino radicals. The modified method has, therefore, a number of points in its favor, since many samples of pathological urine contain crystalline triple phosphate.

### IV. ADDENDUM.

Shortly after the completion of this work, Gill and Grindley published a paper entitled "Total Nitrogen Determination by Kober Method." They found that the Kober method sometimes gave low results for total nitrogen as compared with the official Kjeldahl method. Since most of their low results were obtained in the analysis of plant materials containing relatively large proportions of magnesium and phosphorus, Gill and Grindley concluded that ammonio-magnesium phosphate was formed during the digestion of such materials, and that the nitrogen in such phosphate was not completely removed from triple phosphate crystals by the Kober method. They base this latter conclusion partly on our observation<sup>2</sup> that sodium carbonate is inadequate for complete liberation of ammonia from triple phosphate.

In this connection the reader should be reminded of the fact that ammonio-magnesium phosphate crystals will not form in the

<sup>1</sup> Gill and Grindley: *Journ. Amer. Chem. Soc.*, xxxi, p. 1249, 1909.

<sup>2</sup> Steel and Gies: *this Journal*, v, p. 71, 1908.



ence of an excess of sodium hydroxide. Furthermore we proved<sup>1</sup> that sodium hydroxide, even in small proportions, is able completely to eject ammonia from triple phosphate in the aëration process in the cold. The data in this paper further emphasize that fact. Therefore, some other reason will have to be advanced for the non-removal of nitrogen by the Kober method from the organic materials studied by Gill and Grindley. Kober<sup>2</sup> himself has lately called attention to these facts.

I wish to express my gratitude to Professor William J. Gies for the invaluable assistance he gave me in connection with this investigation.

<sup>1</sup> Steel and Gies: *loc. cit.*

<sup>2</sup> Kober: *Journ. Amer. Chem. Soc.*, xxxii, p. 690, 1910.

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# THE PRESENCE OF ARGININE AND HISTIDINE IN SOILS.<sup>1</sup>

(PLATE I.)

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All plants contain nitrogenous compounds which on the decay of the plant tissues find their way into the soil, forming a part of its organic matter. While the nitrogenous constituents of the plants have been investigated and considerable light shed upon their chemical nature, the organic nitrogenous constituents of the soil have been shrouded with mystery and much controversial matter is found in the literature on this subject. This has been due to the fact that no definite nitrogenous compounds were obtained from the soil in such investigations owing largely to experimental difficulties involved. Recent work in this laboratory has shown that the decomposition of protein and other nitrogenous substances proceeds in the soil much as it does under other conditions and that the products of cleavage and change of these nitrogenous bodies occur in the soil and can be isolated therefrom by appropriate methods.

All protein bodies give some diamino-acids on decomposition. Three of these occur very frequently and usually together, but in varying proportions, one predominating in one protein and another predominating in a protein from some other source. These are lysine, arginine and histidine, all basic compounds of known constitution. Two of these, arginine and histidine, have been isolated from soils.

*Histidine* ( $C_6H_9O_2N_3$ )—Histidine and arginine usually occur together and the method of their isolation depends on the fact that

<sup>1</sup> Published by permission of the Secretary of Agriculture.

they are precipitated by silver salts, sulphate or nitrate, from alkaline solution. The solution may be made alkaline with ammonia or barium, but the fact that the histidine silver precipitate is dissolved by a slight excess of ammonia makes the use of barium hydroxide preferable. Separation of the two is accomplished by taking advantage of the fact that histidine silver is precipitated in slightly alkaline solution while the arginine compound is not precipitated except in a strongly alkaline medium. The isolation and separation of these two compounds thus form simply two steps in one method.

The soil, Houston clay, was treated with 2 per cent sodium hydroxide solution, well-shaken or stirred, and allowed to stand for several hours, the clear liquid syphoned off and acidified with sulphuric acid. In the case of this soil, which was calcareous, the alkaline extract was light-colored and on acidifying the humus precipitate was slight. The acid filtrate was made neutral and to it, without filtering, silver sulphate in excess was added and then filtered. From this point the method of Kossel and Kutscher<sup>1</sup> was used. The neutral filtrate was saturated with barium hydroxide and the precipitate removed and washed with barium hydroxide solution. The precipitate of arginine and histidine silver was then suspended in dilute sulphuric acid and decomposed with hydrogen sulphide. The filtrate from the silver sulphide was then evaporated to a smaller volume, neutralized with barium hydroxide and barium nitrate added until all sulphuric acid had been precipitated. The filtrate from barium sulphate was then concentrated and silver nitrate added until a drop added to an excess of barium hydroxide solution gave a yellow precipitate. The solution was then neutralized with barium hydroxide and the addition carefully continued until a portion of the liquid no longer gave a precipitate with ammoniacal silver nitrate, showing complete precipitation of histidine. The arginine remains in the solution.

The histidine silver precipitate, after being filtered off, washed and suspended in dilute sulphuric acid, was decomposed with hydrogen sulphide. The filtrate from the silver sulphide was freed from sulphuric acid with barium hydroxide and excess of the latter removed with carbon dioxide. The filtrate from barium carbonate was evaporated to dryness, taken up with silver nitrate solution and a drop of nitric acid and filtered from any insoluble matter present. Ammoniacal silver nitrate was then added, the precipitate filtered off, washed and decomposed on the filter paper with dilute hydrochloric acid. The filtrate from the silver chloride contains the histidine as hydrochloride and on concentration to a small volume the histidine crystallizes out as the dihydrochloride in characteristic glassy plates or prisms. The crystals formed are well developed, even when but

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<sup>1</sup> *Zeitschr. f. physiol. Chem.*, xxxi, p. 166, 1900.

small quantity of histidine is present, and can easily be separated and purified by recrystallizing from dilute hydrochloric acid. The crystals are quite characteristic and a crystallographic description has been given by Kossel<sup>1</sup> and Schwantke.<sup>2</sup>

The characteristic form of the crystals and the method by which the compound was obtained are sufficient to establish its identity as histidine dihydrochloride and can be confirmed by two other reactions. Knoop's color reaction: To a solution of the histidine salt add bromine water until the yellow color produced is permanent. On heating, the color disappears but is replaced immediately by a faint red which gradually passes to a deep red, amorphous particles separating, and the liquid becoming turbid. This reaction is not very delicate and is interfered with by the presence of free alkali or excess of bromine. Pauly's diazo reaction:<sup>3</sup> Histidine or its salts in alkaline solution give with diazotized p-toluidine a red color which does not disappear on dilution. Tyrosine also gives a similar color, but histidine or its salts cannot be confused with tyrosine in crystalline form, the latter crystallizing in needles that are nearly insoluble in water. This reaction is much more delicate than the one with bromine, one part in one hundred thousand is said to give a distinct pink color.

The histidine dihydrochloride obtained from the soil gave both the color reactions, the latter very definitely.

The histidine dihydrochloride obtained by this method is easily soluble in water, crystallizes without water of crystallization and melts at 231° C. with some charring below this point. The free base can be obtained from the dihydrochloride by decomposition with silver sulphate and liberation of the base with barium hydroxide. It is not very soluble in water and crystallizes in plates or needles. It is faintly alkaline in reaction, but does not evolve carbon dioxide.

**Arginine,  $C_6H_{14}O_2N_4$ .**—The method of isolating arginine, which is simply a further step in the method used in the isolation of histidine, was as follows:

The filtrate from the histidine silver precipitate was saturated with powdered barium hydroxide, the precipitate filtered off, washed with

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*Zeitschr. f. physiol. Chem.*, xxii, p. 182, 1896.

*Ibid.*, xxix, p. 491, 1900.

*Ibid.*, xlii, p. 508, 1904.

barium hydroxide solution, suspended in dilute sulphuric acid and decomposed with hydrogen sulphide. The filtrate from the silver sulphide was freed from sulphuric acid with barium hydroxide and the filtrate from excess of barium with carbon dioxide. The filtrate from the barium carbonate was neutralized with nitric acid and evaporated to a small volume where arginine nitrate crystallized out. The neutral nitrate,  $C_6H_{14}O_2N_4.HNO_3.H_2O$ , thus obtained crystallizes from water in opaque masses composed of minute needles. When anhydrous it melts at about  $175^{\circ}C.$ , but not sharply. It is easily soluble in water, easily in hot alcohol, but with difficulty in cold. An acid nitrate,  $C_6H_{14}O_2N_4.2HNO_3$ , can be obtained by evaporating the neutral nitrate with excess of nitric acid. It crystallizes without water of crystallization in long needles or plates and melts at  $145^{\circ}C.$  The free base can be obtained by adding silver nitrate solution to the acid nitrate solution and then treating this with sodium hydroxide solution where arginine silver is precipitated. This is decomposed with hydrogen sulphide. The filtrate from the silver sulphide on concentration deposits the free base in rosette-like masses of plates melting at  $207^{\circ}C.$

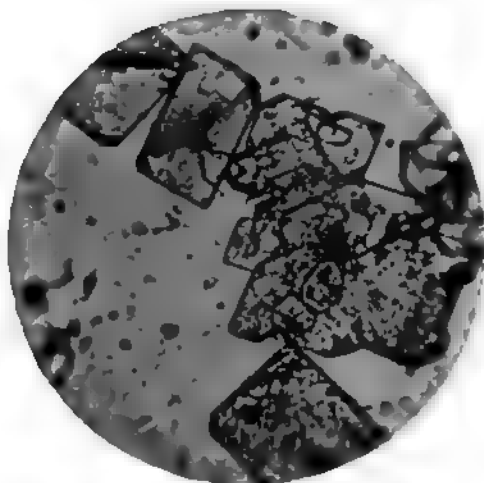
The arginine obtained from the soil as neutral nitrate by the method described was found to conform to the description of this salt<sup>1</sup> as did also the acid nitrate and free base made from

The method by which it was obtained and this conformity of crystalline form, melting point and other properties, are sufficient to establish the identity of the compound obtained as arginine.

The quantity of arginine obtained from the Houston soil was much smaller than the quantity of histidine and histidine has been obtained from several other soils from which no arginine could be obtained.

The isolation of histidine and arginine from soils has several points of theoretical interest. They are basic compounds capable of neutralizing acids. The compounds resulting from the decomposition of organic matter are commonly thought of as essentially acid in character and it is true that practically all that arise from carbohydrates are acid in character, as are also the major products arising from the decomposition of fats. However, the great majority of those derived from the decomposition of proteins while not actually basic as are arginine and histidine, are, with nitrogenous, potentially basic in that they contain the amino  $NH_2$  group. Even when the acid character predominates as in monamino acids they can unite with strong acids to form salts.

<sup>1</sup> Gulewitsch: *Zeitschr. f. physiol. Chem.* xxvii, p. 189, 1899.



PHOTOMICROGRAPH OF THE DICHLORIDE OF THE HISTIDINE OBTAINED FROM  
SOIL





# PYRIMIDINE DERIVATIVES AND PURINE BASES IN SOILS<sup>1</sup>

(PLATE II)

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In earlier papers the presence of protein decomposition products in soils was reported and it was shown that in soils the changes occurring in the decay of such plant and animal materials as enter into the soil are similar to the changes occurring under other conditions in the laboratory or in nature. The presence of arginine and histidine in soils was definitely established by isolating the degradation products of proteins from soils.

In the present paper will be found a further report on the isolation of other nitrogenous compounds from soils, pyrimidine derivatives and purine bases, resulting from the decomposition of nucleoproteids present in the plant and animal remains that enter their way into the soil. The compounds isolated are the pyrimidine derivative, cytosine, and the purine bases, xanthine and hypoxanthine.

In addition to protein which breaks up into amino acids, there is in the cell nucleus what are known as nucleoproteids. These nucleoproteids are combinations of protein with a non-protein radical. This non-protein radical known as nucleic acid is a complex body of unknown constitution, but its decomposition products are well known. These are: Phosphoric acid, a characteristic and constant product; pentose sugar, usually present; lactic acid, sometimes present; pyrimidine derivatives, always present; and purine bases, always present. These last two groups

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are nitrogenous compounds and differ in several respects from the amino-acids. They contain no carboxyl, they are of ring formation and the nitrogen may be either uncombined with hydrogen, or combined as  $\text{NH}$  or  $\text{NH}_2$ .

The plant nucleic acids studied are as yet few in number. That of yeast is the best known and has been extensively investigated by Kossel.<sup>1</sup> Osborne and Harris<sup>2</sup> have isolated a nucleic acid from the wheat embryo which seems to be identical with yeast nucleic acid. Klinkenberg<sup>3</sup> obtained from palm cake and poplar seed nucleic acid resembling that of yeast in composition and there is every reason to conclude that in plants as in animals all parts rich in cells are rich in nucleic acids.

In addition to the nucleic acids of higher plants which may make up a portion of the organic matter of soils, the microorganisms which are so abundant in soils contain and probably elaborate nucleoproteids from other nitrogenous material. Galeotti found nucleoproteids in bacteria and Stutzer<sup>4</sup> has shown that 40 per cent of the nitrogen of molds is nuclein nitrogen.

There are, then, two ways in which nucleic acids and their decomposition products may become part of the soil: From the introduction of the nucleic acids of higher plants on the dead and decay of the same in the soil; and from nucleoproteids elaborated in the soil from other nitrogenous compounds by microorganisms.

The pyrimidine derivative cytosine was discovered by Kossel and Neumann<sup>5</sup> among the products resulting from treating yeast nucleic acid with sulphuric acid. Its constitution was determined by Kossel and Steudel<sup>6</sup> and it was made synthetically by Wheeler and Johnson,<sup>7</sup> and Wheeler and Jamieson.<sup>8</sup> The closest

<sup>1</sup> *Zeitschr. f. physiol. Chem.*, iii, p. 284, 1879; iv, p. 290, 1880; vii, p. 1882.

<sup>2</sup> *Ibid.*, xxxvi, p. 85, 1902; *Journ. Amer. Chem. Soc.*, xxii, p. 379, 1900.

<sup>3</sup> *Zeitschr. f. physiol. Chem.*, vi, p. 155, 1882.

<sup>4</sup> *Ibid.*, xxv, p. 48, 1898.

<sup>5</sup> *Ibid.*, vi, p. 572, 1882.

<sup>6</sup> *Ber. d. deutsch. chem. Ges.*, xxvii, p. 2215, 1894.

<sup>7</sup> *Zeitschr. f. physiol. Chem.*, xxxvii, p. 177, 1902; xxxvii, p. 377, 1903; xxxviii, p. 49, 1903.

<sup>8</sup> *Amer. Chem. Journ.*, xxix, p. 492, 1903.

<sup>9</sup> *Ibid.*, xxxii, p. 342, 1904.

related compounds uracil,  $C_4H_4O_2N_2$ , and thymine,  $C_5H_6O_2N_2$ , are also obtained by the decomposition of nucleic acids. Cytosine, while obtained from nucleic acids of various origin, seems to take the place of thymine in those of vegetable origin so far as they have been investigated. In addition to being obtained from yeast nucleic acid, it has been obtained from the nucleic acid of the wheat embryo.<sup>1</sup>

The nucleic acids are not split into the pyrimidine derivatives and other decomposition products by the proteolytic enzymes such as pepsin and trypsin, but this cleavage can be brought about by erepsin and other closely related enzymes which have been called nucleases.<sup>2</sup> Microorganisms also effect a similar decomposition.<sup>3</sup> From this it would seem that the decomposition products of nucleic acids should be of as constant occurrence in soils as nucleoproteids are in plants.

The purine bases, xanthine, hypoxanthine, adenine and guanine, occur as such both in plant and animal tissues, and are also always derived from the breaking down of nucleoproteids.

Xanthine was first found in a urinary calculus in 1817 and since then has been found to be widely distributed in the tissues of both plants and animals. It has been found present as such in yellow lupines, sprouts of malt, in sprouts of lupines, in *Vicia sativa*, in cucurbita, in phaseolus and in tea leaves,<sup>4</sup> and no doubt further investigations will disclose its presence in many other plants.

Hypoxanthine was first discovered in the spleen and muscles of the heart and like xanthine has been found to be widely distributed in plants and animals. It has been found in the juice

<sup>1</sup> Wheeler and Johnson: *Amer. Chem. Journ.*, xxix, p. 505, 1903.

<sup>2</sup> Nakayama: *Zeitschr. f. physiol. Chem.*, xli, p. 348, 1904. Iwanoff, *Ibid.*, xxxix, p. 31, 1903. Sachs: *Ist die Nuklease mit dem Trypsin identisch*, Inaug. Dissert. Heidelberg, 1905.

<sup>3</sup> Schittenhelm and Schröter: *Zeitschr. f. physiol. Chem.*, xli, p. 284, 1904.

<sup>4</sup> Salomon: *Dubois Arch. f. Physiol.*, pp. 166, 361, 1881; *Bot. Jahr.*, i, p. 290, 1880; Schulze: *Landw. Jahr.*, xii, p. 912, 1883; *Landw. Versuch.*, xlv, p. 383, 1895; Monozzi: *Ber. d. deutsch. chem. Ges.*, xxi, Ref., p. 619, 1888; Baginsky: *Zeitschr. f. physiol. Chem.*, viii, p. 395, 1884; Kossel: *Ibid.*, xiii, p. 298, 1889.

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of potatoes, in the juice of sugar beets, and together with xanthine in most plants where that compound has been found.<sup>1</sup>

Xanthine and hypoxanthine may therefore get into the soil on the death and decay of plants containing them as such, but probably the most constant source of these compounds is to be found in the decomposition of nucleic acid or nucleoproteids in the soil.

The purine bases are susceptible to the action of certain enzymes and of microorganisms. It was shown by Schittenhelm and Schröter<sup>2</sup> that putrefactive bacteria, especially the coli-bacillus, were able to convert one purine base into another. In the animal organism it has been shown by Burian<sup>3</sup> that xanthine and hypoxanthine are oxidized to uric acid by an oxidizing enzyme. Guanine and adenine cannot, however, be oxidized directly in this way, but must first be deaminized by enzymes of another character, being converted thereby to xanthine and hypoxanthine, respectively.<sup>4</sup> The same transformation of guanine and adenine into xanthine and hypoxanthine has been observed by Schindler<sup>5</sup> in putrefaction.

On the other hand, Kutscher<sup>6</sup> found that in the sterile autodigestion of yeast, xanthine and hypoxanthine soon disappeared, leaving only guanine and adenine.

It would seem then that the purine bases are very susceptible to change one to another through the activity of enzymes or microorganisms. Since investigation has shown some of these bases in the majority of soils examined for them, it may be that further investigation will establish some relation between the presence of some one of these bases and the presence of some particular microorganism or combination of biological factors.

The nitrogenous compounds thus far isolated from soils are arginine, histidine, cytosine, xanthine, hypoxanthine, and picoline carboxylic acid. These represent the diamino acids or hexone

<sup>1</sup> Schulze: *Landw. Versuch.*, xxvii, p. 111, 1883; *Landw. Jahr.*, xii, p. 912, 1883; Lippmann: *Ber. d. deutsch. chem. Ges.*, xxix, p. 2645, 1896.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, xxxiz, p. 203, 1903.

<sup>3</sup> *Ibid.*, xliii, p. 497, 1905.

<sup>4</sup> Jones and Austrian: *Ibid.*, xlvi, p. 110, 1906; this *Journal*, iii, p. 227, 1907.

<sup>5</sup> *Zeitschr. f. physiol. Chem.*, xiii, p. 432, 1889.

<sup>6</sup> *Ibid.*, xxxii, p. 66, 1901.

bases, imidazole derivatives,<sup>1</sup> pyrimidine derivatives, purine bases, and pyridine derivatives.

*Cytosine* ( $C_4H_5ON_3 \cdot H_2O$ ).—A crystalline compound identified as the pyrimidine base cytosine has been isolated from several soils by the following method:

The soil was treated for several hours with 2 per cent sodium hydroxide solution, allowed to stand and the dark-colored extract syphoned off. This was acidified with dilute nitric acid and filtered from the humus precipitate. The acid filtrate was then neutralized with sodium hydroxide and filtered from the precipitate formed. The neutral filtrate was then treated with an excess of mercuric nitrate and the whole brought to neutrality again with sodium hydroxide. The precipitate formed was filtered off, washed and decomposed by hydrogen sulphide. The filtrate from the mercuric sulphide was concentrated to a small volume and silver nitrate added in slight excess and filtered from the colored precipitate formed. To the filtrate dilute ammonia was carefully added until a precipitate was no longer formed. An excess of ammonia was avoided since the precipitate is dissolved thereby. The silver precipitate was separated by filtration, washed and suspended in hot water and decomposed by hydrogen sulphide. The filtrate from the silver sulphide was concentrated to a small volume and allowed to stand, when crystals formed in a short time.

These crystals were in the form of needles or thin shining plates with uneven faces. The compound was purified by recrystallizing several times from water. Thus prepared, the compound is in the form of clear crystals which on exposure to the air lose water and become opaque. It does not melt at  $300^{\circ}C$ . but darkens slightly. It is difficultly soluble in cold water, but readily in hot from which solution it rapidly separates on cooling. The relative solubility in hot and cold water is influenced by impurities which generally accompany the first crude separation. It is difficultly soluble in alcohol and insoluble in ether. The water solution is neutral in reaction, but compounds of definite crystalline appearance are formed with mineral acids. The sulphate crystallizes in needles and the hydrochloride in prisms. Addition of sodium picrate to a water solution forms a difficultly soluble picrate crystallizing in needles. A difficultly soluble chloroplatinate is

<sup>1</sup> Histidine, although often considered with the diamino acids, is an imidazole derivative according to the work of Pauly: *Zeitschr. f. physiol. Chem.*, xlii, p. 513; Knoop and Windaus: *Beitr. z. chem. Physiol. u. Path.*, vii, p. 144; viii, p. 406; and Knoop: *Ibid.*, x, p. 111.

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formed, crystallizing in characteristic prisms. Addition of potassium bismuth iodide to an acidified water solution forms a microcrystalline precipitate.

These reactions, the crystalline appearance of the compound and the method by which it was obtained, establish its identity as cytosine. The identification was further confirmed by the following determinations:

A pure preparation crystallized from water, dried a short time between filter paper, lost water of crystallization at 100°C.

	Calculated for $C_4H_5ON_3 + H_2O$ :	Found:
H <sub>2</sub> O.....	13.97 per cent	13.79 per cent

It was found to contain nitrogen corresponding to the formula for cytosine:

	Calculated for $C_4H_5ON_3$ :	Found:
N.....	37.84 per cent	37.92 per cent

These figures are sufficient to make the identification certain.

A modification of the method by which cytosine was obtained that has some advantages is as follows:

Acidify the alkaline soil extract with sulphuric acid, filter from the heavy precipitate, and to the acid filtrate add a solution of mercuric sulphide in 5 per cent sulphuric acid, using an excess. Filter from any precipitate that may be formed immediately and allow the filtrate to stand several days. The precipitate formed on standing should contain cytosine. The precipitate after being filtered off and washed is decomposed with hydrochloric sulphide and the filtrate treated with silver nitrate as in the method first described.

The cytosine can also be obtained from the neutral solution obtained as in the first method without the preliminary precipitation with mercuric nitrate by treating the neutral solution with silver nitrate in the manner described. In this case, however, the final solution contains histidine, if any is present in the soil. Histidine does not interfere with the crystallization of the cytosine but other unknown impurities accompany the two which mercuric nitrate precipitation removes.

The soil from which cytosine was isolated in the manner described was the Marshall loam, a soil that has been described in connection with the isolation of other compounds. Crystals similar

appearance and properties have been isolated by the same method from several other soils and the indications are that pyrimidine derivatives are present as part of the organic matter of many soils.

*Xanthine* ( $C_5H_4O_2N_4$ ).—An extract of the soil was made in the manner used in the isolation of a number of other compounds, by treating with 2 per cent sodium hydroxide.

The dark-colored extract after allowing the soil to settle was syphoned off and the alkaline liquid treated according to the method of Balke<sup>1</sup> for the estimation of purine bases. Fehling's solution, 10 cc. to each liter, was added and the solution heated to boiling. A few drops of a dilute solution of hydroxylamine hydrochloride were then added and this continued until the precipitate formed was red in color. The precipitate was separated by filtration, washed and suspended in a fairly large volume of water, brought to boiling, and treated while hot with hydrogen sulphide. The filtrate from the copper sulphide was then concentrated on the water bath. As this solution became concentrated a film or pellicle of white microcrystalline material formed on the surface and when the liquid was reduced to a small volume this was removed by filtration.

The substance so obtained after purification, by solution in hot water and separation on cooling, was a white microcrystalline powder, little soluble in cold water, but easily soluble in aqueous alkalis. A small quantity evaporated with nitric acid left a yellow residue that on addition of sodium hydroxide and heating became yellowish-red and finally purple, the well-known xanthine reaction.

On dissolving the substance in ammonia and adding ammoniacal silver nitrate, a gelatinous precipitate was formed. This was soluble in hot dilute nitric acid, sp. gr. 1.1, and on cooling this solution there was no separation except on very long standing. This behavior with ammoniacal silver nitrate distinguishes xanthine from other members of the group and is used as a means of separation when occurring together.

The substance obtained from the soil formed crystalline compounds with hydrochloric and nitric acids, the latter having the characteristic crystalline appearance of xanthine nitrate. On adding acetic acid to a very dilute solution of the compound in ammonia and allowing to stand several days, groups of thin rhombic plates were obtained, having the characteristic appear-

<sup>1</sup> *Journ. f. prakt. Chem.*, (2), xlvii, p. 537, 1893.



ance of xanthine obtained under these conditions. The method of isolation and the reactions described fix the compound as purine base and identify it as xanthine.

Instead of treating the alkaline soil extract as described, xanthine or other purine bases can be separated with advantage in some cases by treatment of the acid solution resulting from acidifying the alkaline extract and filtering off the humus precipitate. This acid solution made preferably with sulphuric acid is heated to boiling, solution of copper sulphate added and then a small quantity of a saturated solution of sodium bisulphite. The precipitate formed is filtered off and treated in the same manner as was the precipitate obtained with Fehling's solution. The advantage of the use of copper sulphate and sodium bisulphite lies in the fact that they can be used in neutral, acid or alkaline solution.

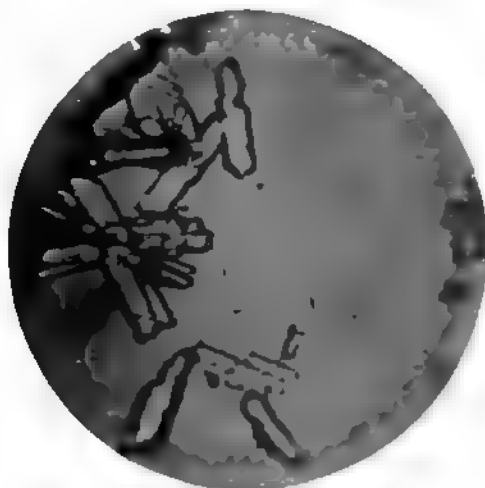
*Hypoxanthine* ( $C_5H_4ON_4$ ).—Hypoxanthine, another purine base, has been isolated from a number of soils by one of the methods outlined in discussing xanthine.

The alkaline soil extract made with 2 per cent sodium hydroxide was made slightly acid with sulphuric acid and filtered from the humus precipitate. Copper sulphate solution was added to the acid filtrate, the solution brought to boiling, and a saturated solution of sodium bisulphite added. The precipitate was separated by filtration, washed, suspended in water and decomposed with hydrogen sulphide. The purine bases separate from the filtrate on concentration. Hypoxanthine, if the only one present, separates only on concentration to a small volume as it is somewhat soluble in water. Its solubility is much influenced by the presence of impurities which accompany it in this procedure and statements regarding its solubility by different authorities are very conflicting.

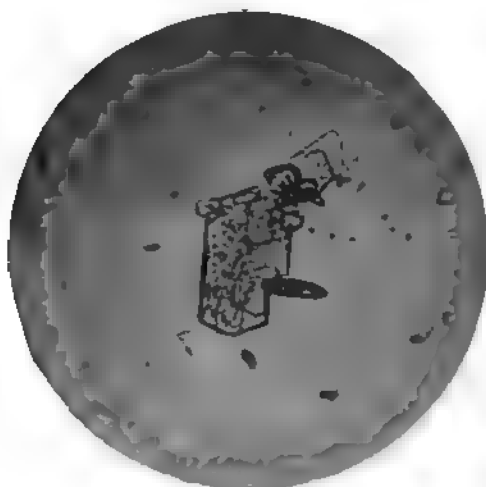
The free base obtained in this way is usually a microcrystalline powder. It forms a hydrochloride of characteristic crystalline appearance, prisms, usually in clusters. It is dissociated by solution in water into the free base and hydrochloric acid.

Hypoxanthine does not give the xanthine reaction with nitric acid and alkalies, but if evaporated with bromine water and nitric acid, a residue is left which turns red on heating with alkalis. Like other purine bases it forms a gelatinous precipitate when ammoniacal silver nitrate is added to an ammoniacal solution and this is dissolved by boiling nitric acid (sp. gr. 1.1) and separated from this solution immediately on cooling in characteristic microscopic needles or plates.





PHOTOMICROGRAPH OF THE HYDROCHLORIDE OF THE HYPOXANTHINE  
OBTAINED FROM SOIL



PHOTOMICROGRAPH OF CYTOSINE OBTAINED FROM SOIL



the identity of the compound obtained from the soil was established as a purine base by the method of its separation and its identity as hypoxanthine proven by the appearance and properties of the free base, the crystalline appearance of the hydrochloride, its behavior with bromine water and caustic soda, and the characteristic crystalline appearance of the silver nitrate compound.



# THE PREPARATION OF CREATININE FROM URINE.<sup>1</sup>

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(Received for publication, September 21, 1910.)

creatinine can be obtained from urine by the picric acid method practically unlimited quantities of urine can be worked up for creatinine in a very short time, and it is now undoubtedly the most suitable source for the preparation of this substance.

The method reduced to its simplest form is as follows: To 8 liters of undecomposed urine in a glass jar is added, with stirring, a hot alcoholic solution of picric acid containing about 10 grams of the acid. The mixture is allowed to settle over

The supernatant liquid is siphoned off and the sediment is roughly washed on a Buchner filter with dilute picric acid solution and finally with cold tap water. The precipitate is transferred to a large beaker (capacity 2 liters) together with 100 cc. of luke warm tap water and about 60 grams of powdered potassium bicarbonate. The beaker is immersed in warm water (55–60°) and carefully stirred until the evolution of CO<sub>2</sub> ceases. The temperature of contents in the beaker should be maintained at 45–50°. If the temperature sinks below 45° the reaction is slow, if it rises above 50° the creatinine is decomposed by heat. Close attention is necessary during this stage of the process for the liquid foams over easily. In order to keep the foam within proper bounds an ordinary mouth spray containing alcohol should be used. By spraying the alcohol into the beaker the foam subsides.

This investigation was completed during the winter of 1907–08.

As soon as the reaction is finished the beaker is put in cold water and is left in a cold place for several hours, or over night. The mixture is filtered and washed with a little cold water. The filtrate is transferred to a large beaker and 50 per cent acetic acid is carefully added until a permanent acid reaction is obtained. At this stage there is again much foaming and the alcohol spray is needed to prevent loss. When the evolution of  $\text{CO}_2$  is practically finished the solution is warmed and an excess of saturated alcoholic zinc chloride solution is added. The familiar double chloride of zinc and creatinine is obtained. The precipitation is usually at an end in one or two days. The precipitate is collected on a Buchner filter and thoroughly washed so as to get rid of the potassium acetate and excess of zinc chloride.

Formerly I decomposed the creatinine salt so obtained by the usual treatment with an excess of lead hydroxide. The method is serviceable for the preparation of creatine as it yields a solution containing about equal parts of creatine and creatinine, which is very nearly free from other impurities. And since the creatine-creatinine mixture remaining after most of the creatine has been removed can be converted into creatinine by a method described in the next paper (see p. 399) this procedure will again become very useful.

From the crude double chloride of creatinine and zinc a new compound having the formula  $\text{Kr}_2\text{SO}_4, \text{ZnSO}_4, 8\text{H}_2\text{O}$ , in which Kr stands for creatinine, is easily prepared. This new salt, which may properly be called creatinine zinc alum, is obtained when the first zinc salt is dissolved in warm 10 per cent sulphuric acid. On adding gradually to the solution acetone, or alcohol and ether, it is almost quantitatively precipitated. Determinations of the water of crystallization, of creatinine, and of sulphuric acid, show the substance to have the composition indicated by the above formula.

This new creatinine compound is serviceable as a starting point for the preparation of pure creatinine. It is easily soluble in water, especially in hot water. By boiling its aqueous solutions with bone-black the adherent coloring matters are removed and from the water-clear filtrates it is again precipitated by the addition of organic solvents. I have prepared several hundred grams of this substance.

From this snow-white product creatinine is prepared as follows: To a 10 per cent solution in hot water the theoretical quantity of barium acetate previously dissolved in hot water is added to precipitate the sulphuric acid, and into the hot mixture is passed hydrogen sulphide to precipitate the zinc. The sulphide and sulphate mixture is removed by filtration, or by means of the centrifuge, and the clear solution which now contains only creatinine and acetic acid is evaporated in a vacuum, at a temperature of about 50°C., to dryness. The residue is washed with a little cold alcohol to remove the remaining acetic acid. The yield of creatinine obtained is practically theoretical.





## PREPARATION OF CREATININE FROM CREATINE.

By OTTO FOLIN AND W. DENIS.

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pure creatine can be obtained rather easily it would be to be able to use it as a starting point for the preparation of creatinine. Recent investigations from several laboratories have shown that by heating creatine with dilute mineral acids it is possible to convert it quantitatively into creatinine for analytical purposes. No one, however, has yet shown how to apply this for the preparation of pure creatinine in dry crystallized form. The method described below does not involve the use of mineral acids or of any solvent. For the transformation of creatine into creatinine we make use only of the water of crystallization of the former, and heat. The dry creatine is converted into crystalline creatinine.

The procedure is as follows: Creatine is transferred to a glass bottle. The closed bottle is placed inside an ordinary cooking jar, the lid of which is held down by a clamp. This is then placed in water in an autoclave, and heat is applied until a pressure of 4.5 kilos per sq. cm. is developed. This pressure is maintained for three hours. After being cooled and opened, the crystals in the bottle are found to consist entirely of coarsely crystalline creatinine.

The mixture may be a little less white than the original substance, and small amounts of ammonia are apt to form. For purification, the substance is either washed a few times with small quantities of cold alcohol, or it is boiled a few minutes with a very small quantity of absolute alcohol and then washed with a little alcohol. About 90 per cent of the theoretical yield of creatinine, consisting of large crystals and assaying 99-100 per cent, is obtained in this way.

Anhydrous creatine does not yield creatinine when subject to the same treatment. In the presence of a little added moisture, one drop of water for each gram of creatine, the transformation into creatinine proceeds a little more rapidly, but the reaction is then accompanied by the formation of more coloring matter and more ammonia. It is a curious and somewhat unexpected fact that the ammonia generated does not seem to interfere with the transformation.

The creatine used as starting material need not be free from creatinine. Indeed any mixture of creatinine and creatine can be subjected to this treatment and be converted into creatinine. Other impurities such as mineral acids or inorganic salts must of course be removed as they will otherwise remain in the final product. The method can be applied to large or small quantities of material with excellent results, as the following two experiments show.

Twenty-five grams of crude creatinine zinc chloride were decomposed in the usual manner with lead hydroxide. From the clear solution was obtained a precipitate weighing 13.5 g. The colorimetric assay showed this to be a mixture of 40 per cent creatine and 60 per cent creatinine. After heating two one-half hours in the autoclave, and washing the product with alcohol, 10.5 grams of dry pure creatinine were obtained.

From 100 grams of the same creatinine zinc chloride decomposition in a similar manner we first allowed some of the creatine to separate out by fractional crystallization. After being recrystallized till free from creatinine this fraction weighed 12.4 grams. The mother liquors were then evaporated to a small volume and precipitated by the addition of alcohol, 50.9 grams of a mixture containing 74 per cent creatinine and 26 per cent creatine were obtained. After heating in the autoclave and washing with alcohol, we obtained 46.8 grams of pure creatinine.

## DETERMINATION OF TOTAL SULPHUR IN URINE.

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In a recent paper Benedict<sup>1</sup> has suggested the use of an oxidizing mixture consisting of copper nitrate and sodium or potassium chlorate for the determination of total sulphur in urine; and in the paper are given a series of results on the determination of sulphur in standard solutions of pure cystin and parallel determinations of total sulphur in twelve urines by the copper nitrate and Folin's sodium peroxide method which would tend to show in the hands of its originator the new method is capable of giving accurate results for total sulphur in urines in a fraction of the time usually required for this determination. An attempt to apply the Benedict method (made at the suggestion of Professor Benedict) has, however, resulted in utter failure, as, owing to the violent decrepitation and spattering produced at the beginning of the reaction by the sodium or potassium chlorate employed, mechanical losses invariably resulted in every one of the forty attempts made. A great amount of care in heating seemed to lessen the spattering, and the addition of a drop of a concentrated solution of cane sugar, recommended by Benedict was also without avail. As Benedict makes the statement that the oxidation can be accomplished with copper nitrate alone and that the addition of an alkaline chlorate is simply made to slow up the reaction and produce a more perfect ash I at first attempted to obtain results by igniting and standard solutions of cystin with copper nitrate but was unable to obtain results much lower than the theory.

5 cc. of urine were evaporated to dryness with 5 cc. of a solution containing 1 gram of copper nitrate in 100 cc. and the residue then ignited accord-

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<sup>1</sup> *Journal of Biological Chemistry*, vi, p. 362, 1909.

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ing to Benedict's directions. Result: (a) = 0.1140; (b) = 0.1150 gm.  $\text{BaSO}_4$  from 100 cc. urine. The same urine gave by the Folin method (a) 0.1286; (b) 0.1290 gm.  $\text{BaSO}_4$  from 100 cc. urine. Ten cc. of a solution of cystin in dilute hydrochloric acid (containing 1.2594 gm. cystin in 250 cc.) gave on ignition with 5 cc. of the copper nitrate solution: (a) 0.0947; (b) 0.0953 gm.  $\text{BaSO}_4$ . Theory = 0.0978 gm.  $\text{BaSO}_4$ .

It would therefore appear necessary to introduce some modification of Benedict's method so as to prevent the too rapid evolution of the oxides of nitrogen. For this I have used sodium chloride with good results.

The method as modified is as follows: To 25 cc. urine contained in a porcelain evaporating dish of approximately four and a half inches in diameter, add by means of a pipette or burette 5 cc. of a solution containing 25 grams of copper nitrate (crystals), 25 grams of sodium chloride and 10 grams of ammonium nitrate. Evaporate to dryness on the steam bath or with a very small flame, then heat gently with a small flame, gradually increasing the flow of gas until the dish is heated to redness, and continue to heat at the latter temperature for ten to fifteen minutes.

Allow to cool and add 10 to 20 cc. of 10 per cent hydrochloric acid. On gently warming for a few minutes a clear solution is obtained.

Transfer to a 200 cc. Erlenmeyer flask, make up to 100 or 150 cc. with water, heat to boiling and add drop by drop 25 cc. of a 10 per cent solution of barium chloride. Let stand one hour or more and filter on a tared Gooch crucible.

A blank must be run on 10 cc. of the oxidizing solution as copper nitrate generally contains traces of sulphate, and the amount of sulphate thus found deducted in the final calculations.

By the use of the above described oxidizing mixture the spattering which is so prominent a feature in the Benedict method is entirely eliminated, the only precaution necessary being the avoidance of too rapid heating at the beginning of the fusions. If at this time the burner be turned up so rapidly that clouds of nitrous oxide gas are evolved the result will be low. The method as above given was first tested on a solution of pure cystin.

1.2594 grams of cystin prepared from wool was dissolved in 20 cc. of normal hydrochloric acid and the solution thus obtained made up to 250 cc. with water.

Eight 10-cc. portions of this solution gave the following results: The figures represent grams of  $\text{BaSO}_4$  from 10 cc. of cystin solution.

0.0981	} Theory = 0.0978
0.0976	
0.0971	
0.0976	
0.0973	
0.0983	
0.0975	
0.0976	

The following results were obtained from twelve urines, in which, in order that we might have some standard for comparison total sulphur has also been determined by Folin's<sup>1</sup> sodium peroxide method.

GRAMS  $\text{BaSO}_4$  FROM 100 cc. URINE

URINE NO.	BY $\text{CuNO}_3$ METHOD	BY FOLIN'S $\text{Na}_2\text{O}_2$ METHOD	URINE NO.	BY $\text{CuNO}_3$ METHOD	BY FOLIN'S $\text{Na}_2\text{O}_2$ METHOD
1.....	{ .076 .076	.070 .074	7.....	{ .093 .091	.093 .098
2.....	{ .061 .061	.062 .067	8.....	{ .089 .085	.089 .090
3.....	{ .080 .080	.085 .086	9.....	{ .102 .103	.099 .101
4.....	{ .155 .155	.153 .152	10.....	{ .130 .130	.128 .129
5.....	{ .082 .082	.080 .087	11.....	{ .416 .416	.420 .402
6.....	{ .074 .074	.070 .078	12.....	{ .620 .611	.617 .620

In conclusion I have to thank Professor Folin for many suggestions made during the course of this work.

<sup>1</sup>This *Journal*, i, p. 155, 1906.



## THE ESTIMATION OF UREA.

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the purpose of the present paper to discuss three methods of estimation, viz., (1) the Benedict-Gephart method, including certain modifications of this process which have been advocated by recent investigators, (2) the Folin method, and (3) a new method for the determination of urea in urine.

### THE BENEDICT-GEPHART METHOD.

November, 1908, Benedict and Gephart<sup>1</sup> published a paper describing the use of the autoclave in connection with the determination of urea in urine. Two alternative procedures were presented.

In (a) the urine was treated with 5 cc. of dilute hydrochloric acid in a test tube, the tube placed in an autoclave and heated to 150–155° for ninety minutes. The tube was then removed from the autoclave, the contents washed into an 800 cc. Erlenmeyer flask, diluted to about 400 cc., treated with 20 cc. of 10 per cent sodium hydroxide solution and distilled into an excess of standard acid. In the second modification (b) the procedure was the same, save that 20 grams of crystallized magnesium sulfate were introduced into the tube before placing it into the autoclave. It was pointed out by Benedict and Gephart that procedure (a) gives slightly higher urea values than does Folin's method for the same samples of urine, while procedure (b) exactly reproduces results obtained by the Folin method. Benedict and Gephart stated that it was their intention to make a further

Benedict and Gephart: *The Journal of the American Chemical Society*, 30, 1760, 1908.

study of the relative merits of the two procedures which they suggested. Shortly (four months) after the appearance of the paper by Benedict and Gephart, Wolf and Osterberg<sup>1</sup> published a communication in which they conclude that the Benedict-Gephart method is inaccurate for the estimation of urea in urine, due to the fact that uric acid and creatinine yield ammonia when subjected to this process. The detailed figures reported by Wolf and Osterberg cannot, unfortunately, be taken as applying to the Benedict-Gephart process, in either modification, for Wolf and Osterberg did not follow exactly the directions for carrying out the process which they were examining. In all their experiments the temperature was between five and ten degrees lower than Benedict and Gephart called for, while in many instances the quantity of acid employed was twice as great as Benedict and Gephart used. It is possible that these two changes in the technique of the process compensated for each other, and that the percentage decompositions reported really apply to the Benedict-Gephart process, but there is no evidence that such is the case. The general conclusion of Wolf and Osterberg in this connection, that the Benedict-Gephart process decomposes more uric acid and creatinine than does the Folin method and is therefore not suitable for accurate work, is unquestionably correct, having been substantiated by Levene and Meyer,<sup>2</sup> and Gill, Allison and Grindley.<sup>3</sup> Furthermore, the present writer's work upon this subject amply confirms the main contention of these investigators. It is interesting to note that while Wolf and Osterberg ascribed the error in the Benedict-Gephart process as due to the acid employed, Gill, Allison and Grindley conclude that the main source of error lies in the strong alkali used. The work of the present writer indicates that the source of error in the method is two-fold, *i.e.*, partially due to the acid, and partly to the alkali. The greater portion of the error is, however, apparently due to the acid, for if magnesium chloride be added *after* the digestion in the autoclave, but prior to the distillation, results, while slightly lower than in the absence of the magnesium salt, are still invariably

<sup>1</sup> Wolf and Osterberg: *Journal of the American Chemical Society*, **xxi**, p. 421, 1909.

<sup>2</sup> Levene and Meyer: *Ibid.*, **xxxi**, p. 717, 1909.

<sup>3</sup> Gill, Allison and Grindley: *Ibid.*, **xxxi**, p. 1078, 1909.



er than in the Folin method. This fact indicates that the magnesium salt plays a peculiar rôle in preventing the decomposition of additional urinary constituents during the digestion in autoclave. Since this is the case, we must conclude that the balance of favor would lie with the Folin method, not only over the Benedict-Gephart method, but even over the modifications of this process which have been reported as giving duplicates for normal urines with the Folin method. The Gill, Allison and Wiley modification decomposes more uric acid than does the Folin process, and the fact that the difference by the two methods is slight in ordinary urines as to be negligible, is no justification for the use of the method in metabolism work, where the uric acid or other unknown decomposable products may be increased, either relatively or absolutely. So long as a method has been shown to be accurate upon pure substances to have the advantage in accuracy, it is hard to find justification for employing a less accurate method in work involving unknown factors. This same objection applies to the modification suggested by Levene and Meyer. In other words, the balance of favor must lie with the magnesium salt method so long as these can be shown to be actually more correct. Slightly altering conditions until normal urines give duplicates with the Folin process, does not make the method methodically correct, or justify its employment in metabolism work where a high degree of accuracy is desirable. If we are to retain a method which is highly accurate, and at the same time avoid the difficulties attending the Folin process, it would appear that such a method must be based upon a solution of the problem of why the Folin method is accurate. This problem is discussed in detail in a later portion of this paper.

#### THE FOLIN METHOD.

As a preliminary to the work reported in the third section of the present paper it was considered necessary to know the limits of accuracy of the Folin method, at least in so far as regards possible decomposition of the more important nitrogenous urinary constituents other than urea. It may at first appear that an examination of the Folin method would be superfluous, but a careful perusal of existing literature will show that such is not the

case. The work of Folin<sup>1</sup> and of Mörner<sup>2</sup> upon this point was done while the technique of the Folin method was still undergoing important modifications. The recent work of Wolf and Osterberg<sup>3</sup> in which they conclude that the Folin method yields absolutely no nitrogen from creatinine or uric acid is open to serious objection. Wolf and Osterberg used, apparently, excessively small quantities of uric acid and of creatinine. They stated that they employed 0.01 cc. of uric acid and of creatinine, which is obviously impossible. Assuming that they employed 10 milligrams, it is plain that 1 per cent of nitrogen yielded by either of these compounds would have represented only about 0.08 cc. of  $\frac{N}{10}$  acid. It is evident then that such quantities of these compounds would be excessively small, and no positive conclusions concerning the absolute accuracy of the Folin method should be drawn from Wolf and Osterberg's work in this connection.

Since then, the Folin urea method appears to have had no adequate examination as to the limits of its accuracy after the introduction of the technique now employed, the present writer deemed it desirable to make such examination.<sup>4</sup>

Folin first proposed his method for urea estimation in 1901 and has modified and discussed the procedure in several subsequent communications.<sup>5</sup> The technique adopted in the present work was that detailed in Folin's latest published communication upon the subject, with the following slight modifications. The digestions were run very vigorously for a period of one and one-half hours. During the first hour the heating was always so great that the indicator changed color at least once a minute, and the heat was but slightly diminished during the last half-hour. It is only by the use of this technique that the present writer has succeeded in obtaining satisfactory results upon all urines en-

<sup>1</sup> Folin: *Zeitschr. f. physiol. Chem.*, xxxii, p. 504, 1901; xxxvi, p. 333, 1902; xxxvii, p. 548, 1902-03; also *Amer. Journ. of Physiol.*, xiii, p. 45, 1905.

<sup>2</sup> Mörner: *Skand. Arch. f. Physiol.*, xiv, p. 297, 1903.

<sup>3</sup> Wolf and Osterberg: *loc. cit.*

<sup>4</sup> My attention was first attracted to this point when testing the autoclave method, by happening to try a mixture of urea and uric acid with the Folin method, and obtaining appreciably higher figures than for the same urea solution alone by the Folin method.

<sup>5</sup> Folin: *loc. cit.*

countered. It is very easy in the use of the Folin method to obtain results which are far too low if the heating be not sufficiently vigorous. In the present work carbon dioxide was removed from the distillates by passing a brisk current of air through the solution for a period of five minutes. Where a blast (or good suction) is available this method of removing carbon dioxide is very convenient. Repeated trials have shown it to be entirely effective, even for saturated solutions of the gas. The final titrations were made using congo red as indicator, instead of lizarine red, as recommended by Folin. In all the experimental work recorded in this paper the standard solutions were of  $\frac{N}{10}$  concentration. The acid used was sulphuric, the alkali, ammonia. The former was standardized against pure sodium carbonate, and the ammonia adjusted until it corresponded to the acid to within .1 per cent. Very frequent control titrations were carried out to insure the accuracy of these solutions. The volumes employed were measured from accurate burettes, the same instruments being employed throughout the entire work. All manipulations involved were executed with the greatest care, and no result is recorded in the present paper which has not been obtained in duplicate at least six times.

The results of the work with the Folin method are summarized in Table 1. The procedure was to add a known quantity of substance to 5 cc. of pure urea solution, the nitrogen content of which had previously been determined, as indicated, by both the Folin and the Kjeldahl method. The creatinine employed in experiment 3 was prepared from different samples of creatine obtained from muscle,<sup>1</sup> through hydrolysis with normal sulphuric acid for forty-eight hours, the acid being subsequently removed by treatment with an excess of barium carbonate. The creatinine content of the resulting solution was determined, and measured volumes of this solution were used in the experiments. As is indicated by the figures in the table, the digestions carrying the creatinine invariably showed slightly more nitrogen than do their controls. The percentage of nitrogen obtained from creatinine

<sup>1</sup> For these samples of creatine I am much indebted to Dr. W. D. Richardson of the laboratory of Swift and Co., Chicago; and to Dr. John F. Lyman, Ohio State University.

in the Folin method is obviously too small to be of account in urine work, but it is of theoretical interest to know that this compound does undergo a slight decomposition during the magnesium chloride method.<sup>1</sup>

The figures for uric acid show a higher percentage yield of nitrogen during the Folin process than do those for creatinine, though still the quantity of nitrogen obtained is so small as to be negligible in most urine work. Mentzel and Arnold<sup>2</sup> some years ago published a communication in which they concluded that the Folin method for urea decomposes appreciable quantities of uric acid, and they cited figures to prove this point. Folin<sup>3</sup> states that he was unable to corroborate the work of these investigators, and explains their yield of nitrogen as due to the use of an impure sample of uric acid. It is to be noted that the present writer's findings of a definite yield of nitrogen from uric acid by the Folin process does not conflict with Folin's failure to obtain nitrogen by his method from the same compound, in as much as the technique employed by the present writer differs very considerably, particularly as regards length of time of digestion, from that which Folin employed.

In order that the present work should not be open to possible objection on the score of the use of an impure sample of uric acid, no pains was spared to secure a pure material. The sample designated as Sample I in the table was an apparently exceptionally pure product supplied by Kahlbaum. For the second set of duplicates a portion of this product was twice recrystallized from boiling distilled water. In the table this is designated as Sample II. It yielded approximately the same quantity of nitrogen by the Folin method as did Sample I. Sample III was

<sup>1</sup> Mörner (*Skand. Arch. f. Physiol.*, xiv, p. 297, 1903) found that small amounts of nitrogen were yielded from creatinine during Folin's digestion. As pointed out above, Mörner's work was done before Folin introduced the use of the indicator into his method, to show when the digestion is becoming alkaline. If, however, as Folin has suggested, alkalinity is the cause of the decomposition of the creatinine, it is not surprising that traces of the compound still yield ammonia in spite of the use of the indicator, for even where the best technique is used, the digesting mixture must be alkaline for brief periods.

<sup>2</sup> Mentzel and Arnold: *Zeitschr. f. physiol. Chem.*, xxxvi, p. 49, 1902.

<sup>3</sup> Folin: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 337, 1903.

TABLE I.

SUBSTANCE EMPLOYED.	PROCESS USED.	TIME OF DURATION.	SUBSTANCE EMPLOYED FOR DISTILLATION.	NITROGEN FOUND.	UREA NITROGEN PERCENT.	EXCESS OF NITROGEN FOUND OVER UREA NITROGEN PRESENT.	NITROGEN FOUND IN ADDED COMBUSTIBLES.
		Min-utes		Gram	Gram	Gram	Per Cent
urea sol'n.	Kjeldahl	60		0.0461			
		80		0.0460			
	Folin	90		0.0461	0.0460		
		90		0.0459			
	Writer	60	$\text{Na}_2\text{CO}_3$	0.0462			
		60	$\text{Na}_2\text{CO}_3$	0.0459			
urea sol'n.	Kjeldahl	60		0.0579			
		60		0.0575			
	Writer	30	$\text{Na}_2\text{CO}_3$	0.0576	0.0578		
		30	$\text{Na}_2\text{CO}_3$	0.0580			
urea sol'n.	Folin	90		0.0464			
sc. + 25		90		0.0463		0.0003	1.2
n. crea-	Writer	90		0.0463			
ne . . .		90		0.0462			
		60	$\text{Na}_2\text{CO}_3$	0.0461	0.0460	0.0001	0.4
		60	$\text{Na}_2\text{CO}_3$	0.0460			
		60	$\text{Na}_2\text{CO}_3$	0.0462			
		60	$\text{Na}_2\text{CO}_3$	0.0461			
		60	$\text{NaOH}$	0.0466			
		60	$\text{NaOH}$	0.0465		0.0005	2.0
urea sol'n.	Folin	90		0.0467	0.0460	0.0006	2.0
2. + 30		90		0.0466			
n. uric							
l. (Sam-							
I) . . .							
	Writer	60	$\text{NaCO}_3$	0.0462			
		60	$\text{NaCO}_3$	0.0461		0.0002	0.6

Estimation of Urea

TABLE I.—Continued.

NO. OF EXP.	SUBSTANCE EMPLOYED.	PROCESS USED.	TIME OF DIGESTION.	SUBSTANCE EMPLOYED FOR DITILLATION.	NITROGEN FOUND.	UREA NITROGEN PRESENT.	EXCESS OF NITROGEN FOUND OVER
			Min-utes		Gram	Gram	G.
5.	2% urea sol'n. 5 cc. + 30 mgm. uric acid. (Sam- ple II).....	Folin	90		0.0466		0.0
			90		0.0464		
		Writer	60	Na <sub>2</sub> CO <sub>3</sub>	0.0462	0.0460	0.0
			60	Na <sub>2</sub> CO <sub>3</sub>	0.0460		
			60	NaOH	0.0466		
			60	NaOH	0.0464		0.0
		Folin	90		0.0468		0.0
			90		0.0465	0.0460	
6.	2% urea sol'n. 5 cc. + 30 mgm. uric acid. (Sam- ple III).....	Writer	60	Na <sub>2</sub> CO <sub>3</sub>	0.0463	0.0460	0.0
			60	Na <sub>2</sub> CO <sub>3</sub>	0.0461		
		Folin	90		0.0563		0.0
			90		0.0564		
7.	2% urea sol'n. 5 cc. + 30 mgm. allan- toin.,.....	Writer	60	Na <sub>2</sub> CO <sub>3</sub>	0.0518		
			60	Na <sub>2</sub> CO <sub>3</sub>	0.0512		0.0
		Folin	90		0.0630		
			90		0.0627		
8.	Human urine, 5 cc. (Sam- ple A).....	Writer	60	Na <sub>2</sub> CO <sub>3</sub>	0.0620		
			60	NaOH	0.0623		
			90	Na <sub>2</sub> CO <sub>3</sub>	0.0622		
			90	NaOH	0.0624		
		Folin	90		0.0630		
			90		0.0627		

TABLE I.—Continued.

NO. OF REP.	SUBSTANCE EMPLOYED.	PROCESS USED.	TIME OF DIGESTION.	SUBSTANCE EMPLOYED FOR DISTILLATION.	NITROGEN FOUND.	UREA NITROGEN PRESENT.	EXCESS OF NITROGEN FOUND OVER UREA NITROGEN PRESENT.	NITROGEN FOUND IN ADDED CONSTITUENT.
			Min-utes		Gram	Gram	Gram	Per Cent
9.	Human urine, 5 cc. (Sample B).....	Folin	90		0.0334			
			90		0.0330			
		Writer	30	$\text{Na}_2\text{CO}_3$	0.0322			
			30	$\text{NaOH}$	0.0323			
			60	$\text{Na}_2\text{CO}_3$	0.0324			
			60	$\text{Na}_2\text{CO}_3$	0.0322			
			90	$\text{NaOH}$	0.0328			
			90	$\text{Na}_2\text{CO}_3$	0.0324			

obtained by recrystallizing a portion of Sample I four times from boiling distilled water. The yield of nitrogen from Sample III is as great as from Sample I. Of all the determinations carried out in this connection, not once did the digestions carrying the uric acid fail to yield appreciably more nitrogen than the controls carrying pure urea.

The results cited in the table with allantoin by the Folin method are directly in line with those reported by Möerner, who found that over 95 per cent of allantoin nitrogen is converted into ammonia during the Folin process. The more continued and vigorous heating employed by the writer in the present work has apparently resulted in a complete decomposition of the allantoin, with a yield of all the nitrogen as ammonia. The allantoin employed was prepared by the writer by oxidation of uric acid with potassium permanganate in the presence of potassium hydroxide. The product was recrystallized seven times from boiling distilled water before employing it in the present work.

The above work upon the Folin method would seem to justify the following conclusions. (1) As at present employed, the Folin method yields traces of ammonia nitrogen from creatinine, the

quantity being under 1 per cent. (2) Using 30 mgm. of the substance, the Folin method gives a yield of about 2 per cent of nitrogen from uric acid. (3) The Folin method for urea decomposes allantoin completely, yielding all of the nitrogen of this compound in the form of ammonia.

#### A NEW METHOD FOR THE ESTIMATION OF UREA.

The fact that the autoclave process, independent of the alkali employed, decomposes additional urinary constituents in greater amount than does the Folin method is well worthy of investigation. Wolf and Osterberg, in the explanation which they offer of the greater accuracy of the Folin method put forth the assumption that the magnesium chloride present during the Folin digestion serves to so decrease the acidity of the mixture, through lowering the solubility of the hydrochloric acid, as well as its dissociation, as to give a quantity of hydrogen ion just sufficient for the hydrolysis of the urea, but not great enough to affect the creatinine or uric acid. This explanation is not adequate to meet all the facts. In their own paper Wolf and Osterberg have shown that acids of low degree of dissociation (acetic and trichloroacetic) may be unable to hydrolyze urea completely in the autoclave, and yet during digestion with these acids creatinine and uric acid suffer very considerable decomposition. Here are acids too weak to hydrolyze urea completely, yet strong enough to decompose uric acid and creatinine. Plainly then, no degree of acidity, under the conditions obtaining in the autoclave could be found which would affect urea and none of the other urinary constituents.

The first step in the solution of the problem appears to lie in the observation that the hydrolyzing agent employed in the Folin method is not an acid at all, but a salt. The fact that hydrochloric acid is formed during the heating and boils off from the mixture is no indication that this acid plays any part in effecting the hydrolysis. The next step in the present study was to attempt hydrolysis of urea in the autoclave, making use of a salt, instead of an acid. The salt employed must obviously be one which will take up the ammonia formed during the digestion. An acid salt cannot be used, for hydrogen ions are to be precluded during the



on. We may however, make use of a neutral salt, the of which yields an insoluble hydroxide, the ammonia being up in accordance with the reaction  $M\text{SO}_4 + 2\text{NH}_4\text{OH} = M(\text{OH})_2 + (\text{NH}_4)_2\text{SO}_4$ . If the metallic hydroxide be completely soluble, and the digestion be carried out in the presence of an excess of the salt, the mixture should remain neutral during the entire period of heating. A study was made of practically all inorganic salts capable of employment in this connection. Those used were the sulphates and chlorides of manganese, cobalt, iron, aluminum, chromium, nickel, copper, mercury, and cadmium. All of these salts, if used in quantities of from 0.1 to 1 gram will serve to convert urea quantitatively into ammonia and carbon dioxide, providing the aqueous solution be heated at 155° for one hour in the autoclave. Each of these salts will, however, cause the hydrolysis of creatinine and uric acid in varying amounts. Some of these salts cause the almost quantitative hydrolysis of uric acid, being far more effective in this respect than hydrochloric acid.

A study of the action of salts as hydrolyzing agents in the digestion of urea in the autoclave served to demonstrate that this procedure cannot be employed with success for the estimation of urea in urine. Since, as has been pointed out above, no salt of any strength whatever can be employed, and no salt can be used with advantage, the conclusion was forced that the autoclave in any modification whatsoever, cannot be employed for accurate estimation of urea in urine.

Since the digesting agent is a salt, yet salts are of no avail in the autoclave. What then, are the conditions which obtain in the autoclave and in the Folin flask? The answer appears to be that during the autoclave heating water is present, while during Folin digestion water is practically absent. Folin, in his paper upon the subject, refers to his digesting mixture as a solution (*Lösung*), and Wolf and Osterberg in speaking of the digestion say that "one has a saturated solution of magnesium chloride." Upon consideration however, it will be obvious that the Folin digestion is actually a melt, a fusion, and not in the ordinary sense a solution. Water is split off at the final point of the fusion and passes off as vapor, but it cannot be present appreciably as a dissociating agent in the contents

of the flask. In the autoclave, on the contrary, water is present in practically infinite quantities throughout the digestion. A consideration of these points will serve to indicate that Folin's process combines two conditions found in no other method for urea estimation so far proposed, viz., (1) the hydrolyzing agent is a salt, and (2) the digestion takes place in the practical absence of water. It will be shown later that both of these factors are probably responsible for the success of the method.

In constructing a method for urea estimation based upon the above considerations the first step was to try heating the dry residue of urea solutions with a salt capable of retaining ammonia. Since water is to be absent there is no objection to the use of a salt here which carries replaceable hydrogen. Potassium bisulphate was the salt which first suggested itself in this connection, and is employed as the main hydrolysing agent in the final method detailed below. Five cc. of 2 per cent urea solution were placed in a wide test tube, and about three grams of potassium bisulphate added. The resulting mixture was boiled almost to dryness, and then the tube was immersed in a sulphuric acid bath at  $160^{\circ}$  for varying lengths of time. The residue in the tube was then washed into a distillation flask, diluted, made alkaline through the addition of sodium carbonate or hydroxide, and distilled into an excess of standard acid. After removal of carbon dioxide from the distillate (where carbonate was employed) the excess of acid was titrated. It was found that where the digestion is carried out for half an hour or more, this procedure gives a constant, and almost quantitative yield of ammonia nitrogen from urea. The following figures are cited as typical of the results in this connection. Five cc. of a 2 per cent urea solution yielded, by Kjeldahl, 0.0459 gm. N. Five cc. of this same solution subjected to the procedure outlined above for 30 minutes yielded (1) 0.0455 gm. N, (2) 0.0452 gm. N. For one determination this slightly lower yield of nitrogen might have been ascribed to experimental error, but repeated determinations showed that the last trace of urea nitrogen could not be obtained by the bisulphate heating, even if the heating were continued for ninety minutes instead of thirty. These results seem to indicate that in presence of bisulphate alone, a very small but perfectly definite quantity of urea undergoes some other rearrangement than into ammonia and

carbon dioxide. Were it simply a matter of lack of complete decomposition of the urea, longer heating or more bisulphate should be effective in obtaining the complete yield of ammonia. As stated above, however, such was not the case, and the other inference seems indicated.

Bearing in mind the point demonstrated earlier in this work, viz., the primary importance of salts in the hydrolysis of urea, the next step was to determine whether addition of a small quantity of a second salt to the bisulphate might not be effective in securing an absolutely quantitative yield of ammonia nitrogen from urea under the conditions described above. A combination of zinc sulphate and potassium bisulphate, employed as described above, will completely hydrolyze urea in one-half hour. Before describing the exact technique to be advocated for the estimation of urea in urine, it is desirable to discuss briefly the alkali to be employed for the subsequent distillation of the ammonia. During work with the autoclave method for the estimation of urea it was found that distillation with sodium carbonate (boiling as usual) invariably gave slightly lower urea values for urine than were obtained where sodium hydroxide was employed. The same result was expected with the new procedure outlined above, but was not found. Human urine yields exactly the same results by the new procedure whether the carbonate or hydroxide be used in the distillation.<sup>1</sup> This fact seems to indicate that in the autoclave digestion a partial decomposition of some substance or substances into compounds other than ammonia occurs, but which yield ammonia upon treatment with the stronger alkali. The new procedure appears to lead to no such partial decomposition, its action being apparently specifically upon urea. With pure uric acid and creatinine the decomposition is slightly less where the carbonate is employed than where hydroxide is used, as is indicated in Table I, but even here the decomposition is scarcely as great where hydroxide is used as occurs in the ordinary Folin method. In either case the percentage decomposition is so low as to be untitratable for 5 cc. of urine, and this explains the fact that

<sup>1</sup> This statement has recently been found to hold also for dog's urine, by Mr. I. Greenwald, working in the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.

results for the urine are identical whichever alkali be employed. Where carbonate is used the distillates must be freed from carbon dioxide before titrating, and while the use of a current of air makes this process easy of accomplishment where a blast is available, still the hydroxide is considerably more convenient to use. Theoretically the carbonate is the correct alkali to be made use of, but until it can be shown that urines yield an appreciably lower figure where this alkali is employed, the hydroxide must be recommended, as being more convenient and rapid.

In the use of the new method a sulphuric acid bath<sup>1</sup> is required which must be kept at a temperature of 162–165°. The arrangement of such a bath is very simple, but for the convenience of those desiring to set up this apparatus it may be of service to describe it in some detail. Where a number of determinations are to be made continuously in a laboratory the following arrangement is to be recommended. Two tall-form Jena-glass or preferably porcelaine<sup>2</sup> beakers of about 800–1000 cc. capacity are two-thirds filled with concentrated sulphuric acid and placed upon an asbestos gauze on a tripod in a hood. A Bunsen burner is placed under the gauze and is connected with the gas inlet by two rubber tubes. One of these tubes first leads through a simple Reichert thermo-regulator, which is immersed in the sulphuric acid bath itself. The second tube, which should be fitted with a spring clamp, is connected directly with the gas inlet. A screen of asbestos should be placed around the burner to prevent disturbing effects from drafts. For preliminary heating up of the bath the spring clamp upon the direct tube is opened and the burner run full-force until the temperature is nearly up to the desired point, when it is slowly closed off and the regulator permitted to keep the bath at the correct degree. One of these baths is kept at 162–165° (not lower), the other at 125–135°. This latter bath is used for preliminary boiling of the tubes to dryness, the former

<sup>1</sup> Glycerine may be substituted for the sulphuric acid in the bath, but has not seemed so satisfactory to the writer. The glycerine gradually dries out, becoming very viscid, and giving off irritating fumes of acrolein during the heating.

<sup>2</sup> For suggesting the use of the porcelain beaker in this connection, which is a very desirable point of technique, I am indebted to Professor Wm. J. Gies of Columbia University.

for the subsequent heating of one hour. Where it is desired, one bath alone may be employed and the thermo-regulator dispensed with. With a little practise one or two adjustments of the flame during the hour of heating will be sufficient to keep the temperature correct. A sketch of the urea apparatus as employed by the writer will be found in Figure I.

The technique recommended for the estimation of urea in urine is as follows. Five cc. of urine are introduced into a rather wide test-tube, and about 3 grams of potassium bisulphate, and 1 to 2 grams of zinc sulphate are added. (The quantities of these salts may be measured roughly. An excess of the zinc salt is to be avoided, as too large a quantity tends to cause slight frothing during the final distillation.) A bit of paraffin, and a little powdered pumice are then introduced into the tube (to prevent frothing and spattering) and the mixture boiled practically to dryness, either over a free flame, or more conveniently, by floating the tube in a bath of sulphuric acid kept at about  $130^{\circ}$ . The tube is then placed in a sulphuric acid bath which is maintained at  $152-165^{\circ}$  (not lower), and left there for one hour. During this heating the tube must be weighted, (a large-sized screw-clamp is convenient, employed as shown in Figure I) so that it will be immersed in the acid for at least three-fourths of its length. At the end of the hour the tube is removed from the bath, the acid washed off under the tap, a little distilled water poured into the tube, and the contents washed (with the aid of heat) quantitatively into an 800 cc. distillation flask. (A small amount of black pigment finally adhering to the sides of the tube may be disregarded, as the ammonium compounds are readily soluble.) The fluid in the distillation flask is diluted to about 400 cc. rendered alkaline through the addition of 15 or 20 cc. of 10 per cent sodium hydroxide (or 25 cc. of 15 per cent sodium carbonate, see preceding discussion of alkali to be employed) and distilled for forty minutes into an excess of standard acid. The residual acid is then titrated, and the urea nitrogen calculated (after subtraction of the previously determined ammonia nitrogen). In dextrose-containing urines this method may be employed in combination with the Förlner-Sjöqvist method.

The results obtained by this method for pure substances and for urine (human) are summarized in Table I. The decomposi-

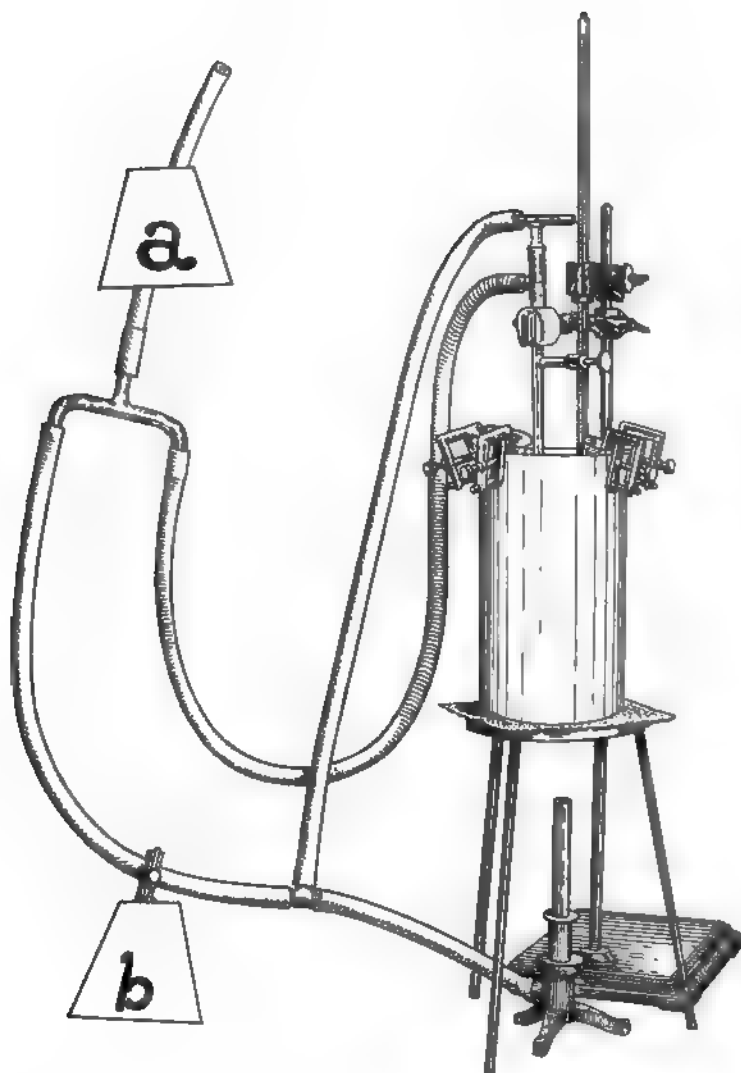
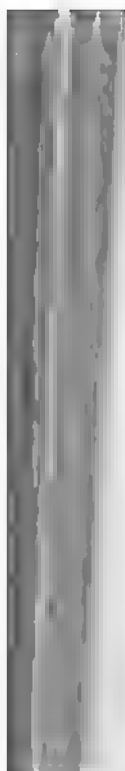


FIGURE I.

The tube *a* is to be connected to the gas inlet. During the preliminary heating-of the bath, screw-clamp *b* is opened, which permits the burner to run at full force. After the thermometer registers about 160°, screw-clamp *b* is closed, so that the thermo-regulator controls the gas inflow. If the apparatus is placed where there are drafts, an asbestos screen about the burner is desirable.

(The negative for this cut was kindly prepared by Dr. Walter H. Eddy of Columbia University).

uric acid and creatinine (using five times the quantity occurring in urine) will be observed to be so low as to be titratable, and a comparison of the figures by the new method and by that of Folin will show that the new procedure requires scarcely half as much ammonia from uric acid, creatinine, and allantoin as does the Folin method. For urines (dog and human) the results for urea nitrogen are either identical by the new method and by that of Folin, or the figures may be slightly lower (1-2 per cent) by the new process. The agreement between the two methods is, however, often as close as two separate titrations by the same method.





## ON BENEDICT'S METHOD FOR DETERMINING TOTAL SULPHUR IN URINE.

By CARL L. A. SCHMIDT.

*from the Hearst Laboratory of Pathology, University of California.)*

(Received for publication, October 8, 1910.)

There has been considerable discussion on the question of determining total sulphur in urine. Folin<sup>1</sup> has suggested the use of sodium peroxide as oxidizing agent in determining total urinary sulphur. Benedict,<sup>2</sup> by the use of copper nitrate and potassium chlorate, obtains results comparable with those of the Folin method. Ritson,<sup>3</sup> by using barium peroxide, obtains somewhat lower values than are given by the sodium peroxide method; however, as stated by Benedict,<sup>4</sup> does not prove the accuracy of his method. Gill and Grindley<sup>5</sup> have likewise obtained higher values than are given by the Folin method, but Folin<sup>6</sup> considers the nitric acid method used by them is less reliable than the Folin method, as described by him. The two methods which have given check results are the sodium peroxide method and the copper nitrate method of Benedict.

I have recently engaged in making a large number of determinations of sulphur in the urines of subjects who were given various doses of sulphurous acid (eating sulphured peaches) and of sodium sulphate, using the Benedict method, the opportunity was offered for check determinations when the urine contained abnormally large amounts of sulphur, but also for making further comparisons with the method of fusing with sodium peroxide.

<sup>1</sup>: this *Journal*, i, p. 155, 1905.

<sup>2</sup>: *Ibid.*, vi, p. 363, 1909.

<sup>3</sup>: *Biochem. Journ.*, iv, p. 337, 1909.

<sup>4</sup>: *Ibid.*, vii, p. 101, 1909.

<sup>5</sup>: Gill and Grindley: *Journ. Amer. Chem. Soc.*, xxxi, p. 52, 1909.

<sup>6</sup>: *Ibid.*, xxxi, p. 284, 1909.

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Benedict's method gave results which not only checked with themselves but also checked with the peroxide method, as can be seen from the following figures. Twenty-five cc. of diluted urine were used in each determination, and 8-10 cc. of the oxidizing solution added. In both methods, barium chloride was slowly added to the hot solution and the precipitate allowed to stand twenty-four hours in using the copper nitrate method and for eight hours in the peroxide. The determinations were carried out both by myself and other members of the laboratory staff.

### 1. Duplicate determinations of sulphur in urine of subjects eating phured peaches—Benedict's method.

- |                                 |                                 |
|---------------------------------|---------------------------------|
| a. 0.0885 gm. BaSO <sub>4</sub> | b. 0.1060 gm. BaSO <sub>4</sub> |
| 0.0881 gm. BaSO <sub>4</sub>    | 0.1061 gm. BaSO <sub>4</sub>    |
| c. 0.1145 gm. BaSO <sub>4</sub> |                                 |
| 0.1141 gm. BaSO <sub>4</sub>    |                                 |

### 2. Duplicate determinations of sulphur in urine of subjects who were given daily doses of sodium sulphite—Benedict's method.

- |                                 |                                 |
|---------------------------------|---------------------------------|
| a. 0.0854 gm. BaSO <sub>4</sub> | c. 0.1134 gm. BaSO <sub>4</sub> |
| 0.0856 gm. BaSO <sub>4</sub>    | 0.1134 gm. BaSO <sub>4</sub>    |
| b. 0.1066 gm. BaSO <sub>4</sub> | d. 0.0721 gm. BaSO <sub>4</sub> |
| 0.1061 gm. BaSO <sub>4</sub>    | 0.0726 gm. BaSO <sub>4</sub>    |

### 3. Comparative results between the Benedict and Folin methods for determining total sulphur in urine.

- |  |  |
|--|--|
| a. 0.0945 gm. BaSO <sub>4</sub> —Folin | d. 0.0800 gm. BaSO <sub>4</sub> —Folin |
| 0.0947 gm. BaSO <sub>4</sub> —Benedict | 0.0798 gm. BaSO <sub>4</sub> —Benedict |
| b. 0.0897 gm. BaSO <sub>4</sub> —Folin | e. 0.0871 gm. BaSO <sub>4</sub> —Folin |
| 0.0897 gm. BaSO <sub>4</sub> —Benedict | 0.0870 gm. BaSO <sub>4</sub> —Benedict |
| c. 0.0871 gm. BaSO <sub>4</sub> —Folin | 0.0865                                 |
| 0.0870 gm. BaSO <sub>4</sub> —Benedict |  |

The range over which Benedict's method can be used was further tested by determinations on urines to which various amounts of grape sugar and blood serum were added. In cases where glucose was added, check results were obtained by both the Benedict and Folin methods but I was unable to obtain satisfactory results with the Benedict method when blood serum was added in small quantities as 5 cc. per 100 cc. diluted urine. The peroxide oxidation on the other hand, gave check results.

Oxidation of urine by means of copper nitrate and potassium chlorate, when albumin is present, is rather violent and very apt to take place explosively. This is quite generally true with substances containing a high percentage of complex nitrogen compounds. Oxidation with sodium peroxide gives no trouble whatever, the reaction proceeding quietly. On the other hand, the peroxide method is apt to give trouble when large quantities of carbohydrates are present.

The results obtained indicate that Benedict's method can be used in determining sulphur in diabetic urine but not in urine containing appreciable amounts of albumin. Any slight loss due to spattering, which may sometimes take place in oxidizing with copper nitrate, can be prevented by covering the evaporating dish with a watch glass.

The following sulphur values were obtained with urines to which glucose and blood serum were added.

- a. 0.3 gm. glucose per 25 cc. diluted urine
  - 0.0945 gm. BaSO<sub>4</sub>—Folin
  - 0.0947 gm. BaSO<sub>4</sub>—Benedict
- b. Both determinations made by Benedict's method.
  - 0.6 gm. sugar added.....0733 gm. BaSO<sub>4</sub>.
  - Same urine—no sugar added.....0733 gm. BaSO<sub>4</sub>.
- c. Diluted urine containing 5 per cent blood serum—peroxide method.
  - 0.1370 gm. BaSO<sub>4</sub>
  - 0.1373 gm. BaSO<sub>4</sub>



# THE DETERMINATION OF THE AMID NITROGEN IN PROTEINS.

By W. DENIS.

*From the Biochemical Laboratory of the Harvard Medical School.)*

(Received for publication, October 19, 1910.)

Up to the present time practically all the quantitative determinations of the ammonia set free from proteins by acid hydrolysis have been made by distilling with magnesium hydroxide, a method originally proposed for this purpose in 1873 by Nasse.<sup>1</sup> In the earlier work the distillation was conducted at 100°; it has soon been shown,<sup>2</sup> however, that this produced variable results, the action of the alkali more ammonia was split off than had actually been detached by the acid hydrolysis.

For this reason Hart has distilled the product resulting from acid hydrolysis of proteins with barium carbonate at 100° and obtained values for ammonia lower than those given by distillation with magnesium hydroxide.

It has also been shown by Embden that solutions of cystin when heated with magnesium hydroxide at 100° yield ammonia; Embden<sup>3</sup> therefore in his work on the Hausmann method recommends that the distillation with magnesium hydroxide should be conducted at reduced pressure and at a temperature not higher than 100°.

On the other hand Osborne, Leavenworth and Brautlecht<sup>4</sup> state that they have made ammonia determination in the product resulting from the acid hydrolysis of several of the vegetable proteins by distilling with magnesium hydroxide, both at atmo-

<sup>1</sup>Nasse: *Arch. f. d. ges. Physiol.*, vi, p. 598, 1872.

<sup>2</sup>Hart: *Zeitschr. f. physiol. Chem.*, xxiii, p. 347, 1901; Folin: *ibid.*, xxxix, 1903.

<sup>3</sup>Emben: *Meister's Beiträge*, v, p. 297, 1904.

<sup>4</sup>Osborne: *Am. Journ. of Physiol.*, xxxiii, p. 183, 1908.

spheric and at reduced pressure, and have by the two methods obtained results which are practically identical.

The method of distilling under reduced pressure has many disadvantages; various pieces of more or less complicated apparatus have been proposed, as elaborate precautions for cooling must be taken in order to avoid loss of ammonia by evaporation. By the reduced pressure distillations much time is also consumed as it is recommended<sup>1</sup> that the operation shall be continued for as long as five hours.

In view of these facts it therefore seemed worth while to attempt to apply the Folin method for the determination of the ammonia in urine to the estimation of this constituent in proteins.

This method which consists primarily of the removal of ammonia from an alkaline solution by a strong current of air, the ammonia so washed out being absorbed in tenth-normal sulphuric acid, has been shown to yield satisfactory results for the estimation of ammonia in urine,<sup>2</sup> blood, Kjeldahl determination<sup>3</sup> and meats.<sup>4</sup>

In order to determine whether ammonia can be quantitatively removed by an air current from a solution of amino-acids, 1 gram of Kahlbaum's casein was boiled for twelve hours with 50 cc. of 20 per cent hydrochloric acid in a flask provided with a reflux condenser; the solution thus obtained was evaporated down to a volume of about 5 cc. to get rid of as much hydrochloric acid as possible, then transferred to a liter distilling flask with about 400 cc. of water and, after the addition of a slight excess of magnesium hydroxide, about 300 cc. of distillate were collected; another portion of water was then added to the residue and the process repeated; after a third addition of water and subsequent distillation, a residue was obtained containing all the amino-acids present in casein, but which may be safely considered to be absolutely free from ammonia as no trace of this substance was present in the last distillate. The residue thus obtained was filtered, acidified with hydrochloric acid, evaporated down to about 5 cc. and

<sup>1</sup> Barker and Cohoe: this *Journal*, i, p. 229, 1905.

<sup>2</sup> Folin: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 161.

<sup>3</sup> Kober: *Journ. Amer. Chem. Soc.*, xxx, p. 1131; Gill and Grindley: *ibid.*, xxxi, p. 1249; Gebellen, Brymelsen and Haevardsholm: *Chem. Zeitung*, xxxiii, p. 793.

<sup>4</sup> Pennington and Greenlee: *Journ. Amer. Chem. Soc.*, xxxii, p. 561, 1910.

transferred with water to an areometer cylinder such as is described in Folin's original paper; to the solution (volume about 20 cc.) was then added a known amount of a standard solution of ammonium sulphate, 1 gram of potassium carbonate, and 2 or 3 cc. of toluol; ammonia-free air was then passed through for two hours, the ammonia being caught in tenth-normal sulphuric acid.

Exactly the same procedure has been employed with gelatin and with wool. The results obtained are given below. The ammonium sulphate solution employed contained 0.00220 gram of nitro-

MEDIA USED. AMMONIA-FREE RESIDUE FROM THE ACID HYDROLYSIS OF	SOLUTION OF (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ADDED	NITROGEN CON- TAINED IN THE AMOUNT OF (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ADDED	NITROGEN RECOVERED
	cc.	gram	gram
Casein.....	20.0	0.0440	0.0440
Casein.....	10.0	0.0220	0.0219
Casein.....	5.0	0.0110	0.0110
Gelatin.....	10.0	0.0220	0.0219
Wool.....	10.0	0.0220	0.0219
Wool.....	5.0	0.0110	0.0110

gen in 1 cc. As before stated Embden<sup>1</sup> finds that cystin when distilled with magnesium hydroxide at 100° gives off ammonia (which observation I have confirmed). That this amino-acid in the presence of a solution of potassium carbonate at room temperature (about 25°) splits off no ammonia is shown by the following experiments.

Twenty-five cc. of a solution of cystin in very dilute hydrochloric acid which contained 0.1044 gram of cystin were placed in an areometer cylinder and exactly neutralized with potassium hydroxide, then 2 grams of solid potassium carbonate and 2 or 3 cc. of toluol were added, and a strong current of air passed through for three hours; absolutely no trace of ammonia was found to have been given off.

As, from the results tabulated above, it would appear possible by this procedure to quantitatively estimate ammonia in the mixture of amino-acids resulting from the acid hydrolysis of proteins

<sup>1</sup> Quoted by Gümbel: Hofmeister's *Beiträge*, v, p. 297, 1904.

I have made a few determinations of this substance in Kahlbaum's casein, in commercial gelatin and in a very pure preparation of edestin which has been kindly furnished me by Dr. T. B. Osborne. The method used is as follows: 1 gram of protein contained in a flask provided with a reflux condenser was boiled for twelve hours with 50 cc. of 20 per cent hydrochloric acid, after which treatment no biuret test could be obtained. The acid solution was then evaporated down on the water bath to a volume of about 5 cc. and when cold transferred with as little water as possible to an areometer cylinder; (at this point the volume of the solution was not allowed to exceed 20 cc.). The cylinder was cooled in a bath of melting ice in order to keep the solution from becoming hot during the subsequent neutralization. After adding 2 or 3 drops of a 1 per cent solution of alizerin red, 2 or 3 cc. of toluol, a sufficient amount of 30 per cent potassium hydroxide solution to just neutralize the small amount of hydrochloric acid still present, and 1 gram of potassium carbonate, a rapid stream of ammonia-free air was passed through the liquid. The ammonia was collected in tenth-normal sulphuric acid. The absorption was facilitated by the use of the Folin improved absorption bulb. After the air current had been passed for two hours the acid was titrated back with tenth-normal sodium hydroxide. It was found, as is usually the case with this method, that the largest part of the ammonia is swept out in the first hour. On passing the current through the product resulting from the hydrolysis of 1 gram of casein I obtained the following results:

1st hour ammonia amounting to 0.01554 gm. of nitrogen was collected  
2nd hour ammonia amounting to 0.00042 gm. of nitrogen was collected  
3rd hour ammonia amounting to 0.00000 gm. of nitrogen was collected

As it seemed desirable to determine whether the employment of the foregoing procedure for the estimation of ammonia would in any way interfere with the subsequent use of the solution for the determination of the mono- and diamino nitrogen by the Hausmann<sup>1</sup> method I have also made a few determinations of these fractions using with slight modifications the method suggested by Osborne and Harris.<sup>2</sup>

<sup>1</sup> *Zeitschr. f. physiol. Chem.*, xxvii, p. 95, 1899; xxix, p. 136, 1900.

<sup>2</sup> *Journ. Amer. Chem. Soc.*, xxv, p. 323, 1903.



Osborne and Harris introduced into the Hausmann method a new feature; that of the separate determination of the melanin nitrogen, which they estimated by filtering off the magnesium hydroxide in the residue left after distilling off the ammonia and determining the amount of nitrogen in this precipitate. This method has also been used by Gumbel.

In the alkaline residue left after the removal of ammonia by the air current I have precipitated the melanoid fraction by adding drop by drop 2-4 cc. of a solution of potassium alum saturated at room temperature. The precipitate was filtered off on nitrogen-free paper and washed until the filtrate no longer gave a test for chlorides; the residue and filter paper were then transferred to a Kjeldahl flask and the nitrogen determined. The filtrate and washings were acidified with sulphuric acid, evaporated down to 100 cc. and the diamino fraction precipitated by the method of Osborne and Harris.

The violent bumping which in the Hausmann method is such a disagreeable feature of the Kjeldahl determinations on the mono- and diamino acid fractions may be entirely eliminated by introducing into the digestion flask (which must be placed in an upright position) a tube bent at right angles and drawn out at one end into a fairly large capillary which must touch the bottom of the flask, a gentle blast of air which has been bubbled through concentrated sulphuric acid is then allowed to pass through this tube, by which means the suspended matter is prevented from settling.

By the use of this device I have been able to employ the entire diamino and mono-amino fractions for the nitrogen determinations.

In the following table are given for comparison the results published by Osborne and Harris<sup>1</sup> whose ammonia determinations were made by distilling with magnesium hydroxide at 100°, and those of Gumbel<sup>2</sup> who distilled at 40°; for gelatin the figures of Hausmann<sup>3</sup> and of Wetzel,<sup>4</sup> both obtained by distilling at 100° are included.

<sup>1</sup> *Jour. Amer. Chem. Soc.*, xxv, 323, 1903.

<sup>2</sup> Hofmeister's *Beiträge*, v, p. 297, 1904.

<sup>3</sup> *Zeitschr. f. physiol. Chem.*, xxvii, p. 95, 1899.

<sup>4</sup> *Ibid.*, xxix, p. 403, 1900.

AUTHOR	PROTEIN	AMID NITRO- GEN	MELANIN NITROGEN	BASIC NITRO- GEN	NON-BASIC NITROGEN	TOTAL NITRO- GEN
		per cent	per cent	per cent	per cent	per cent
Osborne and Harris.....	casein	1.65	0.21	3.46		15.62
Osborne and Harris.....	casein	1.58		3.53	10.30	
Gümbel.....	casein	1.64	0.31	4.25	9.82	15.62
Denis.....	casein	1.60	0.24	3.47	10.20	15.63
Denis.....	casein	1.60		3.54	10.11	
Hausmann.....	gelatin	0.25		5.60	9.79	
Wetzel.....	gelatin	0.27		2.95		
Denis.....	gelatin	0.28	0.08	3.00	12.10	15.65
Denis.....	gelatin	0.28		3.03	11.90	
Osborne, Harris.....	edestin	1.86	0.13	5.98	10.68	18.64
Osborne, Harris.....	edestin	1.86	0.11	6.06	10.62	
Gümbel.....	edestin	1.79	0.29	6.50	10.51	
Denis.....	edestin	1.72	0.10			
Denis.....	edestin	1.70	0.14			

According to these results the amount of ammonia found in casein, gelatin and edestin by the Folin method is but little if at all lower than that obtained by distilling with magnesium hydroxide. It must be remembered however that casein contains only 0.06 per cent cystin, edestin 0.25 per cent, and gelatin practically none; it therefore seemed desirable to make parallel determinations by the two methods on some protein rich in cystin. As no such protein was available in pure condition I have used hair and horn shavings. 3.9 grams of cat hair which had been washed in soap and water and subsequently in alcohol and ether were boiled for fifteen hours with 200 cc. of concentrated hydrochloric acid. The solution was then made up to 500 cc. with water and 100 cc. portions used for the ammonia determinations. Five grams of horn shavings were treated in the same way.

The hair gave by distilling with magnesium hydroxide at 100° 1.25 per cent nitrogen as ammonia, and by the Folin method 0.88 per cent. The horn shavings gave by the distillation method 1.02 per cent and by the Folin method, 0.84 per cent.

As no quantitative data are available concerning the amount of ammonia split off from cystin by distillation with magnesium

hydroxide I distilled 0.1044 gram of cystin with 400 cc. of water and a slight excess of magnesium hydroxide; the distillation was extended over two hours, 300 cc. of distillate being collected; in two experiments an average of 0.70 cc. of  $\frac{N}{16}$   $\text{NH}_3$  was found to have been split off. A considerable amount of discussion has centered around the question as to whether the quantity of ammonia split off by boiling a protein with a mineral acid is a constant factor or whether it is dependent on the nature and concentration of the acid used.

Henderson<sup>1</sup> published a series of results obtained by boiling a number of proteins with various concentrations of hydrochloric and sulphuric acid for different lengths of time. According to these observations the quantity of ammonia split off may be increased by as much as 100 per cent by greatly increasing the time of heating. Hart<sup>2</sup> finds the amount of ammonia increased by the addition of sodium sulphate or sodium chloride to the acid used for hydrolysis, which observation has been confirmed by Kossel and Patten;<sup>3</sup> on the other hand Osborne and Harris<sup>4</sup> and Osborne, Leavenworth and Brautlecht<sup>5</sup> consider that the amount of ammonia split off is a constant factor if the hydrolysis be performed under uniform conditions (until the disappearance of the biuret test).

Below are given the results obtained by boiling Kahlbaum's casein and commercial gelatin with various concentrations of hydrochloric acid. One gram of protein and 50 cc. of acid were used for each experiment; after boiling for the desired length of time, the solution was evaporated down to about 5 cc. in those cases in which hydrochloric acid had been used, and then transferred to the areometer cylinder with a little water. Where sulphuric acid had been employed nine-tenths of the calculated amount of a 30 per cent solution of sodium hydroxide necessary to neutralize this acid present was added before evaporation.

<sup>1</sup> *Zeitschr. f. physiol. Chem.*, xxix, p. 47, 1899.

<sup>2</sup> *Ibid.*, xxxiii, p. 356, 1901.

<sup>3</sup> *Ibid.*, xxxviii, p. 42, 1903.

<sup>4</sup> *Journ. Amer. Chem. Soc.*, xxv, p. 324, 1903.

<sup>5</sup> *Amer. Journ. of Physiol.*, xxiii, p. 181, 1908.

Kahlbaum's casein heated with

5 per cent  $\text{H}_2\text{SO}_4$  for 5 hours gave 1.03 per cent amid nitrogen  
10 per cent  $\text{H}_2\text{SO}_4$  for 10 hours gave 1.55 per cent amid nitrogen  
15 per cent  $\text{H}_2\text{SO}_4$  for 15 hours gave 1.60 per cent amid nitrogen  
20 per cent  $\text{H}_2\text{SO}_4$  for 20 hours gave 1.61 per cent amid nitrogen  
20 per cent  $\text{H}_2\text{SO}_4$  for 70 hours gave 1.60 per cent amid nitrogen  
40 per cent  $\text{H}_2\text{SO}_4$  for 40 hours gave 1.60 per cent amid nitrogen  
50 per cent  $\text{H}_2\text{SO}_4$  for 14 hours gave 1.60 per cent amid nitrogen  
Conc.  $\text{HCl}$  for 7 hours gave 1.54 per cent amid nitrogen  
Conc.  $\text{HCl}$  for 20 hours gave 1.60 per cent amid nitrogen  
Conc.  $\text{HCl}$  for 12 hours gave 1.60 per cent amid nitrogen  
Conc.  $\text{HCl}$  for 50 hours gave 1.59 per cent amid nitrogen

In the experiments in which casein was heated with 5 per cent sulphuric acid for five hours, with 10 per cent sulphuric acid for ten hours and with concentrated hydrochloric acid for seven hours, the biuret reaction could be obtained; in all others it was absent. It would therefore seem that in casein at least the amid nitrogen value is a constant quantity provided we boil until the biuret reaction is no longer obtainable. The case with gelatin seems to be somewhat different.

A high grade of commercial gelatin heated with

Concentrated  $\text{HCl}$  for 7 hours gave 0.25 per cent amid nitrogen  
Concentrated  $\text{HCl}$  for 40 hours gave 0.34 per cent amid nitrogen  
20 per cent  $\text{HCl}$  for 12 hours gave 0.28 per cent amid nitrogen  
25 per cent  $\text{HCl}$  for 50 hours gave 0.31 per cent amid nitrogen

Even here it will be noticed that an enormous increase in the time of boiling must be made in order to appreciably modify the results, for in the product of the first experiment in which gelatin was heated with concentrated hydrochloric acid for seven hours a biuret reaction could still be obtained.

I have also made a number of experiments in order to ascertain whether the percentage of amid nitrogen is increased by the addition of sodium chloride to the hydrolyzing acid, but in no case have I been able to detect the increased quantity found by Hart. A mixture consisting of 5.0 grams of gelatin, 1.5 grams of sodium chloride, 18 grams of sulphuric acid, and 36 grams of water was boiled in a flask provided with a reflux condenser for fourteen hours. When cool it was made up to 500 cc. with water and 100 cc. portions taken for the ammonia determinations; 0.40 per cent amid nitrogen was found. On repeating this experiment with the omis-

f the sodium chloride 0.39 per cent amid nitrogen was ed.

grams of casein, 2 grams of sodium chloride and 100 cc. per cent hydrochloric acid boiled together for twelve hours .60 per cent amid nitrogen; the same experiment repeated at the addition of the sodium chloride gave 1.60 per cent nitrogen.







*Ch. Hester*



## CHRISTIAN A. HERTER, M.D.

Christian Archibald Herter died in New York City on December 5, 1910, in the forty-sixth year of his age. In recording this fact on this occasion, it is our desire not to dwell on our grief and sense of loss, nor shall we essay a biographical sketch. We wish, rather, to express in some degree the lasting encouragement and inspiration evoked by his life and example. For it is our belief that in this we are attempting that which his own spirit and sympathies would have led him to do in contemplating the lives and efforts of others.

The facts of prime significance in any life, and no one felt this more keenly than he, are the ideals which guide its activities; the steadfast striving after the realization of these ideals, and the sympathetic understanding of others. We are permitted to touch upon such aspects of his character only in their relations to his chosen profession.

Of the ideals cherished by Dr. Herter, none was more potent in determining the direction of his professional activities than the conviction dating from his early student days that the practice of medicine was destined to become based upon exact science, and that it was a great privilege as well as a duty for those able to do so to contribute in such measure as was possible to the attainment of this consummation. This conception was not merely a vision of the remote future, but an ever-present incentive, the power of which can be traced through his whole career, finding expression in a variety of ways. The establishment of a laboratory for the prosecution of researches suggested by the problems arising in his own practice when such aids to the enlightened care of the sick were among the novelties of medical practice in this country was an early manifestation of his efforts to contribute to the realization of this ideal. This laboratory, at first modest in dimensions and scope, developed into a center of much activity in medical research by those working under Dr. Herter's immediate direction, and by many others who, through his generous courtesy,

enjoyed its facilities in pursuing their individual problems and who found in him a sympathetic, experienced and ready counsellor.

He was impelled not merely toward the acquisition and accumulation of knowledge for its own sake, but also toward its utilization and diffusion. He constantly pondered the deductions justified by experimental results and the ways in which they might become useful to mankind. Open and eminently practical expressions of this attitude are the creation of the Herter lecture-ships at the University and Bellevue Hospital Medical College and at the Johns Hopkins University, and the foundation of the JOURNAL OF BIOLOGICAL CHEMISTRY, which owes its existence to his initiative and confident foresight.

In these provisions for promoting the advancement of scientific medicine, Dr. Herter considered not merely their present value. He had a vision of the usefulness they should attain in years to come.

We must be pardoned if we dwell for a moment on the foresight shown by him in founding the JOURNAL OF BIOLOGICAL CHEMISTRY. Before deciding upon the undertaking, Dr. Herter conferred with others, notably Professor Abel of the Johns Hopkins Medical School, as to the wisdom of providing at that time an American medium for the publication of chemical researches in the field of Biology, and the scope which a journal started with this object should assume. A result of these conferences was that he and Professor Abel became joint editors. The scope of the JOURNAL was to include researches of a purely scientific character, but, having in mind the needs of clinical medicine, an effort was also to be made to encourage contributions to the knowledge of the chemical aspects of disease.

The way in which the undertaking should be organized also received careful consideration; for Dr. Herter felt that a journal destined to become the repository of original work in a branch of science should possess indefinite stability. It should not be dependent for its existence upon a too limited number of individuals. He therefore took the necessary steps to incorporate the JOURNAL under the laws of the State of New York; providing a Board of Directors which should have as their sole function the conduct and perpetuation of the publication. These arrangements, so wisely wrought by him, have precluded all question of the future of the JOURNAL.

No enterprise could have possessed a greater appeal to Dr. Herter than that which led to the development of the Rockefeller Institute for Medical Research, and no task could have been more congenial to him than to participate with his personal friends in its organization and growth. From its early inception he could not but perceive the necessity of a hospital which should be an integral part of the undertaking; for the whole conception was an expansion of the work which he had done on a necessarily limited scale on his own initiative. He was happy in being able to see this great institution successfully started. It is tragic that he could not have lived to enjoy the full fruits of the laborious years of preparation.

Among the many charming qualities in Dr. Herter's character was his ready appreciation of others. He took personal pleasure in their achievements and was eager in his efforts to encourage those younger than himself to develop their abilities. He was profoundly interested in the study of the lives of men who had accomplished much for the benefit of mankind and loved to trace the thread of continuity that led them step by step to the culmination of their careers. This philosophical bent lent a peculiar wisdom to his counsels which was quickened by the altruistic quality of his ideals.



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## THE REFRACTIVE INDICES OF CERTAIN PROTEINS:

### III. SERUM GLOBULIN.

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(Received for publication, November 7, 1910.)

change in the refractive indices of salt solutions which results from the addition of 1 per cent of the "pseudoglobulins" of blood has been measured by Reiss.<sup>1</sup> His pseudoglobulins were obtained by fractional precipitation with ammonium sulphate and by prolonged dialysis. Fraction I (Pseudoglobulin I) was precipitated at 32–36 per cent saturation with ammonium sulphate, fraction II (Pseudoglobulin II) was precipitated at 40 per cent saturation. The change in the refractive index of water due to the introduction of 1 per cent of the "Pseudo-I" was .00224, that due to the introduction of the "Pseudo-II" being .00230. Reiss adds that the difference between the two terminations is not sufficient to constitute a basis for distinction between the two globulin-fractions, since it is not greater than that which might have arisen through experimental error.

It seemed of some interest to ascertain whether the mode of precipitation of the serum-globulin has any effect upon the change in the refractive index of a solvent which is brought about by its addition therein. There would appear to be at least two globulins in serum, the one insoluble in distilled water, which is precipitated by dilution of the serum or dilution and the subsequent addition of acetic acid; the other soluble in water, precipitable by saturation with magnesium sulphate after the removal of the insoluble

<sup>1</sup>Reiss: *Beitr. zur chem. Physiol. u. Pathol.*, iv, p. 150, 1903.

fraction.<sup>1</sup> According to Hardy<sup>2</sup> these globulins differ markedly in their phosphorus content, the insoluble globulin containing from 0.07 to 0.08 per cent P, the soluble globulin only a trace (about 0.009 per cent) of phosphorus. The material employed in the experiments described below was the insoluble globulin.

(i) PREPARATION OF THE GLOBULIN.

Three liters of ox-serum were diluted with ten volumes of distilled water, and CO<sub>2</sub> was bubbled through it for about half an hour. The globulin which was thus precipitated was allowed to settle in tall glass cylinders, the supernatant fluid being syphoned off after settling. The precipitate was then washed with about 60 liters of distilled water, in two washings. The globulin was then dissolved in a minimal quantity of  $\frac{N}{10}$  hydrochloric acid and reprecipitated by cautious neutralization with  $\frac{N}{10}$  potassium hydroxide. This precipitate, after settling and the decantation of the supernatant fluid, was washed in 60 liters of distilled water in six successive washings, the precipitate, after each agitation with distilled water, being allowed to settle for 24 hours in the presence of toluol, after which the supernatant fluid was drawn off and the globulin suspended in a fresh quantum of distilled water. The thick suspension of globulin which was thus obtained after the final washing was kept, in the presence of toluol, in a stoppered bottle and used in this form, since globulin, if washed with alcohol and ether and dried is redissolved only with difficulty. The suspension was, of course, always well shaken before withdrawing a sample.

Twenty-five cc. samples of this suspension were placed in small and accurately weighed beakers, the fluid was then evaporated to dryness on a water bath and the residue was dried at 70° over sulphuric acid until its weight became constant. Three determinations yielded the following results:

Determinations:	Grams of Globulin in 100 cc. of Suspension.
1	1.49
2	1.47
3	1.48
	—
Average:	1.48

In all, about 14 grams of globulin were obtained.

<sup>1</sup> Clarence Quinan.: *Univ. of California Publ., Pathology*, i, p. 1, 1903.

<sup>2</sup> W. B. Hardy: *Journ. of Physiol.*, xxxiii, p. 330, 1905.

## (ii) THE REFRACTIVE INDICES OF SOLUTIONS OF SERUM GLOBULIN IN WATER.

In previous communications<sup>1</sup> I have shown that the refractive indices of aqueous solution of casein, ovomucoid, ovovitellin or of the paranucleins are connected with their concentrations by the following formula:

$$n - n_1 = a \times c$$

$n$  being the refractive index of the solution of the protein,  $n_1$  that of the solvent in which it is dissolved,  $c$  the percentage of protein in the solution, and  $a$  a constant, i.e., the change in the refractive index of the solvent which is brought about by the addition of 1 gram of casein to 100 cc.

To 25 cc. of  $\frac{N}{16}$  potassium hydroxide were added 25 cc. of the suspension of globulin and the volume of the mixture was made up to 100 cc. by the addition of distilled water. A similar solution was made up containing 50 cc. of the suspension but this proved to be too opalescent to obtain a satisfactory reading in the refractometer. The following was the result obtained:<sup>2</sup>

TEMPERATURE 23°.

$c$ = Per Cent of Globulin in the Solution.	$n_1$ = Refractive Index of the Solvent ( $\frac{N}{16}$ KOH).	$n$ = Refractive Index of the Solution.	$a = \frac{n - n_1}{c}$
0.37	1.33178	1.33262	0.00227

To 25 cc. of  $\frac{N}{16}$  hydrochloric acid were added 25 cc. of the suspension and the volume was made up to 100 cc. by the addition of distilled water. A solution containing 50 cc. of the suspension was too opalescent to obtain a clear reading in the refractometer. The following was the result obtained:

<sup>1</sup> T. Brailsford Robertson: *Journ. of Physical Chem.*, xiii, p. 469, 1909; *Journ. of Indus. and Engineering Chem.*, No. 10, October, 1909; this *Journal*, vii, p. 359, 1910.

<sup>2</sup> In these, as in previous experiments, a Pulfrich refractometer, reading the angle of total reflection to within 1' was employed. The source of light was a sodium flame.

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TEMPERATURE 23°.

$c$ = Per Cent of Globulin in the Solution.	$n_1$ = Refractive Index of the Solvent ( $N_{15}^D$ HCl).	$n$ = Refractive Index of the Solution.	$a = \frac{n - n_1}{c}$
0.37	1.33170	1.33255	0.00230

These values of  $a$  are identical, within the experimental error of the method. Their average is .00229, which is identical with the value of  $a$  for Reiss's "Pseudoglobulin II."

The fact that different globulin fractions possess so very nearly identical values of  $a$  despite their widely differing phosphorus content presents an interesting analogy to the behavior of the paranuclein group. I have elsewhere shown<sup>1</sup> that the paranucleins which result from the partial hydrolysis of casein can be separated into fractions which differ in their power of neutralizing bases and in their phosphorus content, but which are indistinguishable in the quantitative effect which they have upon the refractive index of their solutions. The "insoluble" globulin is readily converted, by hydrolysis, into the "soluble" form,<sup>2</sup> just as the paranuclein of high phosphorus content is readily transformed, by partial hydrolysis, into the paranuclein ("Paranuclein A") of low phosphorus content. The decomposition which leads to the splitting off of phosphorus from these molecules is not, apparently, very profound, since the density of the molecules is unaltered thereby.

Serum globulin presents a striking contrast to the other proteins in the magnitude of the influence which it exerts upon the refractive index of its solution. The following are the values of  $a$  which have hitherto been ascertained:

VALUES OF  $a$  FOR AQUEOUS SOLUTIONS

Pseudoglobulin II.....	0.00230	(Reiss)
Insoluble Serum Globulin.....	0.00229	(Robertson)
Pseudoglobulin I.....	0.00224	(Reiss)
Crystallized Serum Albumin.....	0.00201	(Reiss)
Amorphous Serum Albumin.....	0.00183	(Reiss)

<sup>1</sup> T. Brailsford Robertson: this *Journal*, viii, p. 287, 1910.  
<sup>2</sup> A. E. Taylor: *ibid.*, i, p. 345, 1906.



Ovomucoid.....	0.00160	(Robertson)
Casein.....	0.00152	(Robertson)
Paranuclein.....	0.00140	(Robertson)
Paranuclein A.....	0.00140	(Robertson)
Ovovitellin.....	0.00130	(Robertson)

THE REFRACTIVE INDICES OF SOLUTIONS OF SERUM GLOBULIN  
IN ALCOHOL-WATER MIXTURES.

In adding alcohol to a solution of potassium globulinate no precipitation occurs even when the concentration of alcohol in the mixture is over 90 per cent. A notable *diminution* in the opalescence of the solution occurs, however, upon the addition of small quantities of alcohol, so that in the presence of 25 to 50 per cent alcohol solutions of potassium globulinate are much clearer and more transparent than equally concentrated solutions in water; solutions in alcohol-water mixtures are also yellower in color than those in water. On progressively adding alcohol to a 0.37 per cent solution of globulin in  $\frac{N}{16}$  potassium hydroxide and 50 per cent alcohol, no alteration in the appearance of the solution occurs until the concentration of alcohol reaches 80 per cent, when an increase in opalescence occurs. This increase is, however, no greater than that which is caused by the addition of an equal volume of distilled water. No *precipitation* occurs upon the further addition of alcohol up to 90 per cent or over.

To 25 cc. of  $\frac{N}{16}$  potassium hydroxide were added 25 cc. of Kahlm's C. P. 99.8 per cent alcohol and 25 cc. of the globulin suspension. The volume of the mixture was then made up to 100 cc. by the addition of distilled water. A similar mixture was made up containing 50 cc. of the suspension;<sup>1</sup> the marked effect of alcohol in clearing solutions of potassium globulinate is seen in the fact that the refractive index of this latter solution could readily be determined. The following were the results obtained:

Due to the volume-contraction which occurs on mixing alcohol and water the volume of this latter mixture was not exactly 100 cc; it was, however, made up to 100 cc. by the addition of a few drops of water.

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TEMPERATURE 23°.

$c$ = Per Cent of Globulin in the Solution.	$n_1$ = Refractive Index of the Solvent ( $\frac{N}{10}$ KOH in 25 Per Cent Alcohol).	$n$ = Refractive Index of the Solution.	$a = \frac{n - n_1}{c}$
0.37	1.34578	1.34652	0.00200
0.74	1.34578	1.34728	0.00203
		Average:	0.00202

To 25 cc. of  $\frac{N}{10}$  potassium hydroxide were added 50 cc. of alcohol and 25 cc. of the globulin suspension<sup>1</sup> and the refractive indices of the solvent and of the solution were determined. The following was the result.

TEMPERATURE 23°.

$c$ = Per Cent of Globulin in the Solution.	$n_1$ = Refractive Index of the Solvent ( $\frac{N}{10}$ KOH in 50 Per Cent Alcohol).	$n$ = Refractive Index of the Solution.	$a = \frac{n - n_1}{c}$
0.37	1.35729	1.35773	0.00119

The influence of serum-globulin upon the refractive index of its solution, therefore, decreases markedly with increasing concentration of alcohol in the solvent. In 25 per cent alcohol the change in the refractive index of the solvent which is brought about by the addition of serum globulin is obviously, within the limits employed, proportional to the concentration of the globulin.

(iv)    THE REFRACTIVE INDICES OF SOLUTIONS OF SERUM GLOBULIN IN ACETONE-WATER MIXTURES.

On adding acetone to a solution of potassium globulinate a notable *diminution* in the opalescence of the solution is observed, similar to that which occurs when alcohol is added. On progressive addition of acetone a very marked increase in opalescence is observed when the concentration of acetone attains about 80 per cent, and

<sup>1</sup>Cf. previous footnote.

between 80 per cent and 90 per cent the globulin is precipitated in flocculent coagula.

To 25 cc. of  $\frac{N}{10}$  potassium hydroxide were added 25 cc. of the globulin suspension. The volume of the mixture was then made up to 100 cc. by the addition of distilled water. A similar mixture was made up containing 50 cc. of the suspension; to make up for volume contraction a few drops of distilled water had to be added to this mixture to make the volume up to 100 cc. The following were the results obtained:

TEMPERATURE 23°.

$c$ = Per Cent of Globulin in the Solution.	$n_1$ = Refractive Index of the Solvent ( $\frac{N}{10}$ KOH in 25 Per Cent Acetone).	$n$ = Refractive Index of the Solution.	$s = \frac{n - n_1}{c}$
0.37	1.34669	1.34753	0.00227
0.74	1.34669	1.34838	0.00227

To 25 cc. of  $\frac{N}{10}$  KOH were added 50 cc. of acetone and 25 cc. of the globulin suspension; to make up for volume-contraction a few drops of distilled water were added to this mixture to make the volume 100 cc. The following was the result obtained:

TEMPERATURE 19°.

$c$ = Per Cent of Globulin in the Solution.	$n_1$ = Refractive Index of the Solvent ( $\frac{N}{10}$ KOH in 50 Per Cent Acetone).	$n$ = Refractive Index of the Solution.	$s = \frac{n - n_1}{c}$
0.37	1.35984	1.36029	0.00146

The influence of serum-globulin upon the refractive index of 25 per cent acetone is therefore, within the experimental error, identical with its influence upon the refractive indices of aqueous solvents and is proportional to the concentration of the globulin. In 50 per cent acetone the effect of the addition of globulin is very much less than it is in aqueous solvents or in 25 per cent acetone.

## CONCLUSIONS.

1. The value of  $a$  in the equation  $\frac{n - n_1}{c} = a$  where  $n$  is the refractive index of the solution of the protein,  $n_1$  that of the solvent, and  $c$  is the percentage concentration of the protein, has been determined for solutions of "insoluble" serum globulin in  $\frac{N}{40}$  aqueous potassium hydroxide,  $\frac{N}{40}$  aqueous hydrochloric acid,  $\frac{N}{40}$  potassium hydroxide in 25 per cent and in 50 per cent alcohol and  $\frac{N}{40}$  potassium hydroxide in 25 per cent and in 50 per cent acetone.
2. The value of  $a$  for "insoluble" serum globulin dissolved in acid or alkaline water is 0.00229; this is, within the experimental error, identical with the value of  $a$  (0.00230) determined by Reiss for "Pseudoglobulin II."
3. The value of  $a$  for insoluble serum globulin dissolved in alkaline 25 per cent alcohol is constant for the range of globulin-concentrations employed and is 0.00202.
4. The value of  $a$  for insoluble serum globulin dissolved in alkaline 50 per cent alcohol is 0.00119.
5. The value of  $a$  for insoluble serum globulin dissolved in alkaline 25 per cent acetone is constant for the range of globulin-concentrations employed and is, within the experimental error, identical with its value in water.
6. The value of  $a$  for insoluble serum globulin dissolved in alkaline 50 per cent acetone is 0.00146.

## A NOTE ON THE NITROGEN METABOLISM OF THE COYOTE (*CANIS LATRANS*, SAY).

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(Received for publication, November 8, 1910.)

In 1905 Swain<sup>1</sup> discovered among the nitrogenous constituents of a coyote's urine a substance to which he assigned the empirical formula,  $C_{12}H_9N_4O_4$ . This substance excited our interest by its alleged resemblance to urocanic acid,  $C_{12}H_{12}N_4O_4$ , a body which, described first by Jaffé<sup>2</sup> in 1874, was for a long time known only as an excessively rare constituent of the urine of the dog,<sup>3</sup> but was found recently by one of us among the products of a long-continued tryptic digest of plasmon.<sup>4</sup> It was with the object of procuring a supply of Swain's substance, and of comparing it directly with the urocanic acid in our possession, that we originally turned our attention to the urine of the coyote.

From this, our main point of view, the investigation has met with only failure. We have had a coyote under observation for nine months. It has been placed under a variety of conditions, at one time deprived of food, at another upon a mixed diet, or upon a pure flesh diet, or again (following a suggestion which we owe to a private communication from Dr. Swain) upon a diet of semi-putrid meat. The urine was sometimes allowed to stand for months in the ice-box, sometimes subjected to the method of isolation described by Swain, sometimes submitted to other processes which

<sup>1</sup> Swain: *Amer. Jour. of Physiol.*, xiii, p. 30, 1905.

<sup>2</sup> Jaffé: *Ber. d. deutsch. chem. Gesellsch.*, vii, p. 1669, 1874; viii, p. 811, 1875.

<sup>3</sup> It has been but twice met with in this situation, first by its discoverer Jaffé, and again in 1898 by Massot, working in the laboratory of Siegfried. See Siegfried: *Zeitschr. f. physiol. Chem.*, xxiv, p. 399, 1898.

<sup>4</sup> Hunter: *Journ. of Physiol.*, xxxvii; *Proc. Physiol. Soc.*, p. xxxvii, 1908; and this *Journal*, vi; *Proc. Soc. Biol. Chem.*, p. xliii, 1908-9.

seemed likely to yield the desired result. In no case, whether the quantity worked up was great or small, did it yield a trace of the substance sought.

The explanation may lie quite simply in the fact that our "coyote" was of a species which is distinct from that of Swain's. He was dealing with the Californian coyote, *Canis ochropus*, Eschscholtz; our animal, on the other hand, had been bred in Wyoming, and was a specimen of *Canis latrans*, Say, probably of the race known as *nebracensis*.<sup>1</sup> Whether this entirely explains our result, or whether some other factor of diet or of individual idiosyncrasy is responsible, it is in the meantime impossible to determine. It is noteworthy that the urine of our coyote differed from that of Swain's in yet another particular—in the absence namely of kynurenic acid.<sup>2</sup> We possess at present no evidence which would justify the suggestion of any connection between the two circumstances.

The observations made during the course of our inquiry furnished us from time to time with data bearing upon the general question of the animal's nitrogenous metabolism. It is these data that are here presented. They are by no means as complete as might be desired, but they are sufficient to illustrate more fully than has yet been done the details of nitrogen excretion in this close relative of the domestic dog.

The subject of the two experiments reported was a female weighing 7.1 kilograms.<sup>3</sup> It was about twelve months old, an age at which the coyote is said to have attained its adult size. As the average weight of females is given at 11 kilograms, ours was an undersized specimen. When it first entered the laboratory, it was suffering from a rachitic affection to which coyote cubs in cap-

<sup>1</sup> For the identification of the species we are indebted to Dr. H. D. Reed of the Department of Vertebrate Zoölogy in this University.

<sup>2</sup> Kynurenic acid was sought by the method of Capaldi: *Zeitschr. f. physiol. Chem.*, xxiii, p. 92, 1897. We had no difficulty in isolating considerable quantities of kynurenic acid from several samples of dog's urine examined by the same procedure.

<sup>3</sup> It was one of two which were presented to this laboratory through the courtesy of Dr. Frank A. Crandall of the Zoölogical Gardens at Buffalo, N. Y. We gladly take this opportunity of expressing to Dr. Crandall our indebtedness for the gift.

tivity are very liable. At the time of our metabolic observations, though exhibiting some resultant deformity of limb, it was in excellent health.

The nitrogen excretion of this animal formed the object of quantitative observations under two conditions: firstly, during a period of nitrogenous equilibrium upon a purely meat diet, and secondly, under the influence of starvation. Throughout each experiment, as well as for several days before it, the coyote was confined in a suitable metabolism cage. The urine was collected as it was naturally passed; in the case of an animal so wild the use of the catheter was out of the question. Fortunately we succeeded in inducing micturition at approximately the same hour every day, so that no very great error is involved in taking the excretion periods to be of equal length. The collected portions of urine were preserved by means of thymol and cold storage. Determinations of total nitrogen, ammonia and urea were made within forty-eight hours; the others as soon thereafter as was possible. All figures recorded, with the exception of those for purine nitrogen, are means of closely agreeing duplicates.

The analytical methods made use of were the following: *total nitrogen*, Kjeldahl-Gunning for urine, for food and feces Kjeldahl-Gunning-Arnold in the convenient modification of Salkowski;<sup>1</sup> *ammonia*, sometimes Folin, sometimes Shaffer; *urea*, Mörner-Sjöqvist-Folin, selected in preference to the simple Folin method, because it is less liable to the error caused by allantoin; *creatine* and *creatinine*, Folin. *Purine nitrogen* was determined in the precipitate obtained by the Krüger-Schmid method from 400 cc. of the mixed urine of several days. The urine was first boiled with 5 per cent sulphuric acid, and the directions given by Schittenhelm<sup>2</sup> for carnivore urines were carefully followed. The quantity of purine-copper compounds so obtained was very small, and separate determinations of the uric acid and purine bases which it contained would have been of little value. Indeed neither the copper nor the silver method led to the separation of any crystallized uric acid; it was represented in each case by a few amorphous brown granules,

<sup>1</sup> Salkowski: *Zeitschr. f. physiol. Chem.*, lvii, p. 515, 1908.

<sup>2</sup> In Abderhalden's *Handb. d. biochem. Arbeitsmethoden*, iii, p. 887, Berlin, 1910.

to reckon which as pure uric acid would obviously have been meaningless. *Allantoin* was estimated by the method of Wiechowski.<sup>1</sup> The substitution of this valuable method for the very inaccurate older ones of Poduschka and Loewi has already materially increased our knowledge of the occurrence and significance of allantoin in mammalian metabolism. As its application will in future be indispensable for all investigations involving allantoin determinations, and as no description of it has yet appeared in English, it may not be out of place here to indicate briefly its principle and the mode in which we applied it.

The method depends on the fact that if urine be freed from ammonia, chlorides, and substances precipitable by phosphotungstic acid and basic lead acetate, and if it be at the same time so far diluted that its urea concentration is less than 1 per cent, then any allantoin it contains can be completely precipitated by mercuric acetate in the presence of much sodium acetate. From the mercury precipitate the allantoin may be recovered in pure crystalline form, identified by its melting point, and weighed; or, if one is satisfied that no impurities have been brought down along with it, it may be reckoned simply from the nitrogen content of the precipitate.

The conditions essential for the complete precipitation of the allantoin may be attained in a variety of ways; we have found suitable in the present instance the one described in Wiechowski's first paper on the subject, and have applied it in the following way. One hundred cc. of the already somewhat diluted urine are measured into a 250 cc. volumetric flask. This is acidified with 10 cc. of 8 per cent sulphuric acid, 10 per cent phosphotungstic acid (Merck) is cautiously dropped in just so long as any precipitate appears, and water is added up to the mark. The flask is set aside for an hour, and its contents thereupon filtered into a separating funnel, or stoppered flask. Lead carbonate is added, in bulk and with constant shaking, until carbon dioxide ceases to be evolved, and the fluid reacts neutral or only slightly acid. This operation, which takes some time, having been completed, the insoluble lead salts are filtered off, and 200 cc. of the filtrate are measured into the 250 cc. flask. This is treated with no more than the necessary amount of basic lead acetate solution and the volume is made up again to 250 cc. After filtering, the lead is removed by hydrogen sulphide and the excess of hydrogen sulphide by a stream of air. Once more 200 cc. of the filtrate are placed in the 250 cc. flask, and the last traces of chloride removed by the careful addition of concentrated silver acetate solution. The flask is filled to the mark, the silver chloride filtered off, the filtrate freed from silver by hydrogen sulphide, and from hydrogen sulphide by a stream of air. The acid fil-

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<sup>1</sup> Wiechowski: *Beitr. z. chem. Physiol. u. Pathol.*, xi, p. 109, 1908; see also *Biochem. Zeitschr.*, xix, p. 368, 1909; xxv, p. 431, 1910; *Arch. f. exp. Path. u. Pharm.*, lx, p. 185, 1909.



trate from the silver sulphide should now give no precipitate with phosphotungstic acid, basic lead acetate or silver nitrate; if on testing small samples it is found to fulfill these conditions, it is ready for the precipitation of the allantoin. Two portions of 100 cc., each representing 25.6 cc. of the original diluted urine, are conveniently taken for this step. They must first be rendered *exactly* neutral to litmus by means of chlorine-free sodium hydrate.

The reagent employed to throw down the allantoin is 0.5 per cent mercuric acetate in half-saturated sodium acetate solution. The bulk of this reagent required may be considerable, 150 cc. or more. If allantoin is present in any but the very minutest quantities, a cloud or precipitate appears at once. Precipitation is, however, complete only after several hours, and it is best to allow the beakers to stand over night at this stage. The precipitates are then collected on nitrogen-free filters, and washed with water. The filtrate should be tested with a few drops of a fresh 0.1 per cent solution of allantoin, with which it should react immediately; one can assure oneself in this way, firstly, that an excess of the reagent is present, and secondly, that the solution has really been freed from all substances that would interfere with the precipitation.

To obtain the allantoin free the mercury precipitate is washed into a beaker or flask, and decomposed by hydrogen sulphide in the heat. As the sulphide separates in a non-filterable condition, the whole is in the first place evaporated to dryness. It is then taken up in warm water, filtered clear through a small double filter, the filter thoroughly washed, and filtrate and washings evaporated. The allantoin crystallizes out; if the crystals are not quite colorless, they can be made so by the addition of a few drops of 3 per cent hydrogen peroxide during evaporation. They are dried at 100°C. and weighed. They generally give the correct melting point without recrystallization.

In the urines we had to deal with this method worked so satisfactorily, that the mercury precipitate contained practically no impurities at all. This is shown, *e.g.*, by the two following analyses of one urine, in each of which the allantoin was determined in one duplicate by the above gravimetric process, and in the other by estimating the nitrogen in the mercury precipitate.

**First Determination:** The amount of the final filtrate taken corresponded to 13.3 cc. of the original urine.

	Allantoin.	Allantoin Nitrogen.
Gravimetrically.....	16.0 mgms.	5.68 mgms.
By Kjeldahl.....	16.2     "	5.74     "

**Second Determination:** The amount taken was equivalent to 25.6 cc. urine.

	Allantoin.	Allantoin Nitrogen.
Gravimetrically.....	32.3 mgms.	11.5 mgms.
By Kjeldahl.....	32.7     "	11.6     "

The total volume of the urine was 500 cc. On calculating its allantoin content from the above data, we get the following results:

Determin- ation.	Method.	Allantoin.	Allantoin Nitrogen.
No. 1	Weighing.....	0.60 gms.	0.21 gms.
	Kjeldahl.....	0.61 “	0.22 “
No. 2	Weighing.....	0.63 “	0.22 “
	Kjeldahl.....	0.64 “	0.23 “

In each case the allantoin isolated was pure; it melted at 231°–232° C., simultaneously with a sample of synthetic allantoin heated alongside of it.

The two determinations quoted were made by different observers and each represents a first trial of the method. Under these circumstances the agreement must be considered sufficiently gratifying; as the technique became more familiar closer approximations could be attained. Already from these first results it appeared that (in the urines we had to deal with) the isolation and weighing of the allantoin could be dispensed with. Accordingly all the allantoin data subsequently recorded were obtained by estimating the nitrogen of the mercury precipitate.

#### FIRST EXPERIMENT—NITROGEN EXCRETION ON A MEAT DIET WITH NITROGENOUS EQUILIBRIUM.

The meat used was lean beef, freed from visible fat, and prepared and preserved according to the plan described by Gies.<sup>1</sup> It contained 3.80 per cent of nitrogen.

Before being placed in the cage the animal had been fed freely on all kinds of scraps, and was in a well-nourished condition. Nevertheless it was some time before it could be brought into approximately exact equilibrium. A daily ration of 150 grams of meat proved quite inadequate, being accompanied by a loss of 3.2 grams nitrogen in five days. Ultimately equilibrium was attained with 250 grams of meat containing 9.50 grams nitrogen. As the coyote weighed throughout the equilibrium period 7.1 kilograms, it was receiving 8.4 grams of protein per kilogram. Dogs are stated to require on a pure flesh diet from 8 to 10 grams protein per kilogram.

<sup>1</sup> Gies: *Amer. Journ. of Physiol.*, v, p. 235, 1901.

The allowance of 250 grams had been fed to the animal for five days before analyses were commenced. During the six-day period which formed the actual experiment it was given in two portions, 150 grams at 11 a.m., and 100 grams at 6 p.m.; and it was combined with 5 grams of bone ash, which, among other purposes, served that of delimiting the feces. Water was allowed *ad libitum*. Each day's urine was collected separately and together with the washings of the cage was made up to 1000 cc.; convenient aliquot parts were taken for the analyses. The feces were also collected daily, and after treatment with a little sulphuric acid were dried at 40° C.; the feces of the whole period were afterwards thoroughly mixed, and their nitrogen determined in a number of samples. Hair and scurf were treated in the same way.

The balance of nitrogen for the period is shown in Table I.

TABLE 1.

	TOTAL.	DAILY AVERAGE.
	gms.	gms.
Intake.....	57.00	9.50
Output—		
Urine.....	53.69	8.948
Feces.....	2.48	0.413
Epidermal waste.....	0.80	0.133
Total.....	56.97	9.495
Balance.....	+0.03	+0.005

The coyote was therefore during this experiment in exact equilibrium. The daily records, showing the partition of the urinary nitrogen among its various forms, will be found in Table II.

TABLE II.

DAY OF EXPERIMENT.	CC. OF URINE.	GRAMS OF NITROGEN.						PER CENT OF TOTAL NITROGEN.																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
		Total.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	Creatinine.	Creatinine.	Urea.	Ammonia.	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om the above figures may be calculated as the average abso-  
amount of each constituent excreted daily:

	grams.
Urea.....	16.65
Ammonia.....	0.36
Creatinine.....	0.27
Creatine.....	0.34
Allantoin.....	0.87

we compare with our results the few corresponding data given  
wain,<sup>1</sup> we find that his coyote (on a diet mainly composed of  
excreted 89.95 per cent of its urinary nitrogen in the form of  
0.26 per cent as allantoin, 0.09 per cent as kynurenic acid,  
0.016 per cent as uric acid. The daily allantoin excretion was  
0.031 to 0.074 gram, but the determinations were made by the  
æowi method, which has been shown to be utterly unfit for  
titative work. The very low figure for uric acid is in agree-  
with our experience as to the difficulty of isolating any uric  
at all from coyotes' urine.

ND EXPERIMENT—NITROGEN EXCRETION DURING STARVATION.

is experiment followed the first after an interval of rather more  
two months. During this time the coyote's diet received no  
vision whatever; it was evidently far from generous, for when  
was at length withheld, the weight of the animal had fallen  
7 kilograms. Starvation found it, therefore, in very meagre  
ition. The fast was one of eight days' duration. As the daily  
ne of urine was very small, the analyses were carried out on  
day portions made up to 300 cc. each. Otherwise the details  
the same as during the first experiment. The animal was  
ved to drink as much water as it wished.  
e results are shown in Table III, while in Table IV the actual  
ngs are halved, so as to show the mean *daily* excretion through-  
he fast.

wain: *loc. cit.*

TABLE III.

*Excretion by periods.*

DAY OF FAST.	CC. OF URINE	GRAMS OF NITROGEN							PER CENT OF TOTAL NITROGEN						
		Total	Urea	Ammonia	Creatinine	Creatine	Allantoin	Purine	"Rest"	Urea	Ammonia	Creatinine	Creatine	Allantoin	Purine
1	60														
2	45	6 335	260	1450	200	060	290	01330	35 83	1 2	3 3	2	0 9	4 6	5 7
3	58														
4	45	5 124	290	1070	180	090	300	01330	14 83	8 2	1 3	5	1 8	5 9	2 7
5	46													0 3	
6	31	4 824	090	0940	170	060	280	01330	11 84	8 2	0 3	5	1 2	5 8	2 3
7	30														
8	28	4 543	840	1010	150	050	290	01330	10 84	6 2	2 3	3	1 1	6 4	2 2

TABLE IV.

*Average daily excretion.*

DAY OF FAST.	GRAMS OF NITROGEN							
	Total	Urea	Ammonia	Creatinine	Creatine	Allantoin	Purine	"Rest"
1-2	3 17	2 63	0 073	0 10	0 03	0 145	0 0067	0 18
3-4	2 56	2 15	0 054	0 09	0 045	0 15	0 0067	0 07
5-6	2 41	2 05	0 047	0 085	0 03	0 14	0 0067	0 06
7-8	2 27	1 92	0 051	0 075	0 025	0 145	0 0067	0 05

## DISCUSSION OF THE RESULTS.

A general inspection of Tables II, III, and IV will show at once that, whether our coyote was in equilibrium or fasting, its nitrogen metabolism did not differ in any essential respect from that of a

og placed under similar conditions.<sup>1</sup> One or two details are possibly worth calling attention to.

*Urea.* On a meat diet the nitrogen excreted as urea follows closely the variations of the total nitrogen, and forms therefore an exceedingly constant proportion of the latter. This proportion is slightly smaller than that found by Swain, but the difference is not greater than may be frequently met with in different individuals of any species. During the starvation period also the percentage value for urea nitrogen remains very steady. As was to be expected, it assumes a lower value than upon the meat régime. At the fall is hardly striking; in the latter half of the period it tends rather to rise again, nor does it at any time reach the low values usually met with in starving dogs. This circumstance is doubtless to be correlated with the peculiar course taken, as we shall presently note, by the ammonia excretion.

*Ammonia.* On the high protein diet this constituent will be seen to assume much the same relative importance as it does in dogs, and its variations about the mean value are not considerable. In man, on the other hand, it exhibits a quite unexpected behavior. While it is true that in carnivorous animals starvation is not so constantly nor so markedly associated with acidosis as it is, *e.g.*, in man, yet some relative increase, great or small, in the ammonia excretion is decidedly the rule. Here we meet with the exactly contrary effect. The proportion of nitrogen eliminated as ammonia is *smaller* than it was on the meat régime; decreasing continuously up to the fifth and sixth days, it reaches at length a value as low as 1 per cent. This diminished importance of the ammonia during starvation was emphasized by analyses of the urine passed on the days immediately preceding and immediately following the fast; on these the ammonia nitrogen formed respectively 3.6 per cent and 1.9 per cent of the whole—figures quite comparable with those of the earlier (equilibrium) period.

Our figures may be compared, for example, with those to be found in the following papers: Underhill and Kleiner: this *Journal*, iv, p. 165, 1908; Richards and Wallace: *ibid.*, iv, p. 179, 1908; and Steel, *ibid.*, v, p. 85, 1908–9. The first-mentioned gives complete analyses for a fast of thirteen days' duration. (The extensive data of Osterberg and Wolf: *Biochem. Zeitschr.*, v, 104, 1907, apply to dietary conditions not imitated in our experiments.)

A finding so unusual seemed to called for confirmation. Accordingly, after about a week of meat feeding, another (three-day) starvation experiment was instituted. During this it was sought to exclude any possibility of a loss of ammonia. Each portion of urine voided was immediately washed quantitatively into a receptacle containing a few cc. of diluted acid; and total nitrogen and ammonia were at once determined in the daily quantities. The analyses showed the same progressive fall as before in the relative amount of ammonia, and the results of the main experiment were confirmed.

Day.	Total Nitrogen.	Ammonia Nitrogen.	
	gram.	gram.	per cent.
1	2.56	0.079	3.1
2	1.80	0.048	2.7
3	1.96	0.051	2.6

As a matter of fact the observation here made is not a completely isolated one. Thus, though a relative increase in urinary ammonia is generally regarded as an invariable accompaniment of fasting in the human subject, E. and O. Freund<sup>1</sup> have reported a case where not only was such an increase not observed, but the ammonia on some days formed as little as 1.1, 1.2, and 1.6 per cent of the total nitrogen; in the case of dogs also a search of the records reveals occasional examples of the same phenomenon.<sup>2</sup> As to the explanation, we can make no definite suggestion. It is of course unlikely that the behavior shown by our animal is normal to all coyotes. It may represent an individual peculiarity, or it may have had a relation to the previous state of nutrition.

*Creatinine.* The daily creatinine of the food period is both absolutely and relatively all but invariable; not only so, but two months later in the fasting condition we find its absolute amount, at least for the first three or four days, to be still exactly the same. In the later days of starvation the quantity eliminated decreases, as it is usually observed to do. The "creatinine coefficient" of our coyote lies between 13 and 14; this is rather higher than usual in the dog, where the value is seldom found above 12.

<sup>1</sup> E. and O. Freund: *Wien. klin. Rundschau*, xv, p. 69 and 91, 1901.

<sup>2</sup> Cf. the dog numbered 405 in Wolf and Osterberg: *Biochem. Zeitschr.*, xvi, p. 476, 1909.



*Creatine.* The very variable creatine excretion of the first experiment is doubtless largely if not entirely exogenous in origin. With 100 grams of meat the coyote must have ingested daily at least 1 gram of creatine.<sup>1</sup> It will be seen that not all of this reappears as urinary creatine, for the daily excretion of that substance averages only a third of a gram, and some of this *may* be endogenous. Some of the food creatine, on the other hand, is converted into creatinine, for the latter is passed in no greater quantity on a meat régime, than when food is withheld. Similar observations have been made on dogs.<sup>2</sup> The occurrence of a variable amount of endogenous creatine in the hunger urine is of course a frequently served phenomenon.

*Allantoin and Purines.* As a consequence in the main of the recent work of Wiechowski,<sup>3</sup> it has come to be recognized that allantoin plays a much greater and more general rôle in metabolism than was formerly assigned to it. It has been shown that it is present, often in quantities not previously suspected, as a regular normal constituent of all mammalian urines thus far examined, and that in every case but that of man it is to be regarded as the principal and often almost the only end-product of the metabolism of the purines. We have found the extent and behavior of the allantoin excretion of the coyote to be practically identical with that of the dog. Thus, e. g., Wiechowski reports that small dogs (3.5 to 5 kilos) eliminate daily during starvation from 0.2 to 0.3 gram allantoin; our animal, weighing about 7 kilos, excreted under the same conditions 0.42 gram. It is found further that the daily allantoin excretion of a fasting dog is a constant value, undiminished even at the end of any days of hunger.<sup>4</sup> The same striking fact is to be observed in

<sup>1</sup> See Emmett and Grindley: this *Journal*, iii, p. 491, 1907.

<sup>2</sup> Cf. Lefmann, *Zeitschr. f. physiol. Chem.*, lvii, p. 476, 1908. In one of Lefmann's experiments a dog, whose urine on a bread-and-milk diet contained only traces of creatine, excreted 1.4 grams as a result of the ingestion of 880 grams of meat. The corresponding amount for 250 grams would have been 0.4 grams, which compares with the 0.34 grams excreted daily by our coyote. The creatinine excretion was unaffected.

<sup>3</sup> Consult the papers already cited.

<sup>4</sup> Schittenhelm, *Zeitschr. f. physiol. Chem.*, lxii, p. 80, 1909, reports a fast of 27 days duration where the allantoin was practically the same on the last day as on the first.

our coyote; throughout an eight days' fast, during which the total nitrogen fell by 28 per cent, the allantoin maintains its original level, period by period, unchanged. No other constituent of the hunger urine exhibits so remarkable a degree of constancy. We are enabled accordingly to fix with precision the value of the *endogenous* allantoin excretion—0.42 gram = 0.15 gram nitrogen; and so far as our observations go they accord with the view of Wiechowski, that in the lower mammals this value is as much a constant, and as truly a measure of the endogenous purine metabolism, as is in man the output of endogenous uric acid.

In the meat-feeding experiment the allantoin excretion was much higher than in starvation, and very much less regular. The increase is of course to be explained by the transformation into allantoin of the purines of the food; the irregularity is doubtless associated entirely with the same process, and is probably merely an indication of irregularities of absorption. The average daily value of this *exogenous* factor in the allantoin output is  $0.31 \text{ minus } 0.15 = 0.16$  gram of nitrogen. Now 100 grams of beef contain 0.063 gram of purine nitrogen;<sup>1</sup> the amount of the latter ingested daily was therefore 0.16 gram. It would seem then that the meat purines *must* have been entirely converted into allantoin. This conclusion, which is quite in accordance with recent work on the metabolism of purines in the dog, receives support from a consideration of *the* excretion of nitrogen in purine form. This is minimal in amount during starvation (0.0067 gram daily), and on the meat diet is *but* slightly greater (0.0088 gram). In the former case, only 4.3 per cent, in the latter 2.8 per cent of the total nitrogen of purine derivation is excreted as urinary purine; while 95.7 per cent and 97.2 per cent respectively appear in the shape of allantoin. Whether, therefore, endogenous or exogenous purines be concerned, it is evident that among the end-products of their metabolism allantoin plays an enormously preponderating part.

Our data are of course not sufficient to do more than illustrate these views. Conclusive proof of their correctness is furnished by recent experiments of Schittenhelm<sup>2</sup> upon dogs. For the relation between allantoin and purine nitrogen Schittenhelm establishes

<sup>1</sup> Cf. Burian and Hall: *Zeitschr. f. physiol. Chem.*, xxxviii, p. 336, 1903.

<sup>2</sup> Schittenhelm: *loc. cit.*

lues that are practically identical with ours, and shows, that either by starvation nor by the ingestion of considerable amounts sodium nucleate is the relation appreciably altered.<sup>1</sup>

Under all circumstances, allantoin claimed from 93 to 96.8 per cent the nitrogen derived from purine. It had already been demonstrated by Lechowski that in the organism of the dog or rabbit uric acid, so far as it differs change at all, is as good as quantitatively converted into allantoin. The production of allantoin from purines or purine-containing material had long since formed the subject of many earlier researches, notably those of Mendel and his collaborators (cf. Mendel and White: *Amer. Journ. of Physiol.*, xii, p. 85, 1904); but until an accurate method of estimation was made available, the quantitative relations involved escaped detection.



## COMPARATIVE ANALYSES OF THE URINE OF THE FOX, DOG AND COYOTE.

By P. B. HAWK.

*(From the Laboratory of Physiological Chemistry of the University of Illinois.)*

(Received for publication, November 8, 1910.)

The purpose of the present study was to make an examination into the characteristics of the urine of the fox, dog and coyote when each of these animals was fed a uniform diet embracing the same constituents and apportioned accurately upon the basis of the body weight of the animal. The actual daily rations as fed are listed in Table I, p. 475. It will be observed that not only were the solid constituents of the diet fed upon a "kilogram per body weight" basis, but the water ingestion was likewise accurately apportioned, inasmuch as certain experiments made by my associates and myself upon the influence of water drinking have demonstrated conclusively that the volume of water ingested alters to a marked degree the metabolic processes of the organism.<sup>1</sup> The animals received 0.6 gram of nitrogen and 26.6 grams of water per kilogram of body weight.

The fox and coyote which were utilized in the experiment were the property of Professor F. L. Charles of this University, whose courtesy in the matter is greatly appreciated. The animals had been members of a private "Zoo" for several years and were therefore more or less domesticated, thus rendering them considerably more satisfactory for metabolism studies than the undomesticated animals. At the commencement of the experiment the

<sup>1</sup> Hawk: *University of Pennsylvania Medical Bulletin*, xviii, p. 7, 1905; Fowler and Hawk: *Journal of Experimental Medicine*, xii, p. 388, 1910; Rulon and Hawk: unpublished; Hattrem and Hawk; unpublished; Mattill and Hawk: Report before American Physiological Society (Boston 1909-10), unpublished; Wills and Hawk: report before American Physiological Society (1909-10), unpublished.

fox (male) weighed 4.7 kilograms and the coyote (female) weighed 15 kilograms. The urine was collected in twenty-four hour specimens as voided by the animals. The methods of analysis used were, Kjeldahl method for total nitrogen, Folin's methods for ammonia and creatinine, and the Folin-Benedict and Myers method for creatine. The tests on each animal covered a period of six days. For comparative purposes a dog was placed on a properly apportioned diet (see Table I, p. 475) and the urine examined during the same period.

*Fox Urine.* Data from the examination of the urine of the fox are given in the upper portion of Table II, p. 476. Attention may be directed to four points in connection with the general data collected from the examination of the daily samples of fox urine. These are the uniformity of urine volume, specific gravity, and total nitrogen and creatinine-nitrogen content of the specimens. It will be seen that the urine voided during the different 24-hour periods varied only from 43 cc. to 65 cc., the specific gravity ranged from 1043 to 1045, the total nitrogen output was found to vary from 1.981 gram to 2.154 grams per day, whereas the creatinine-nitrogen output for the different days fell between the limits of 0.04 gram and 0.05 gram. These data are surprisingly uniform when it is remembered that the animal was not catheterized, but was allowed to urinate spontaneously and the urine voided during the various twenty-four hour periods was subjected to analysis.<sup>1</sup>

More detailed information regarding the fox urine is given in Tables III, IV and V, p. 477. In Table III, for example, we have recorded the percentage distribution of nitrogen in the form of ammonia, creatinine, and creatine. A consideration of these data indicates that ammonia-nitrogen constituted from 1.7 to 3.8 per cent of the total nitrogen output whereas creatinine-nitrogen made up 1.9 to 2.5 per cent while the daily output of creatine accounted for but 0.1 to 1.9 per cent of the daily nitrogen excretion. In Table IV, are presented the *average* data from the urine examinations. It will be observed, in the first place, that the average daily urine volume is rather low. The fox received 125 cc. of water per

<sup>1</sup> Very similar conditions as regard uniformity were noted in one of our studies in which a small dog served as subject. See Howe and Hawk: Unpublished.

clay and excreted on the average but 51 cc. of urine. In other words about 60 per cent of the water is unaccounted for solely upon the basis of urine volume. (This relation between water ingestion and urine volume is more fully discussed on p. 473). The other data tabulated in Table IV, indicate that 2.8 per cent of the total nitrogen excretion was eliminated in the form of ammonia. It is further shown that 2.2 per cent of the nitrogen output was excreted as creatinine whereas but 0.9 per cent appeared as creatine. This preponderance of creatinine-nitrogen over creatine-nitrogen was not observed to exist in the case of the urine of either the dog or the coyote. In fact in the coyote urine the creatine-nitrogen was found to be fully equivalent to that occurring in the form of creatinine.

The average daily data *per kilogram of body weight* are recorded in Table V, p. 477. It is there shown that 10.9 cc. of urine was excreted per kilogram of body weight and that this volume of urine contained 0.442 gram of total nitrogen, 0.012 gram of ammonia nitrogen, 0.01 gram of creatinine-nitrogen and 0.003 gram of nitrogen in the form of creatine. The urine of the fox was not examined for kynurenic acid. Capaldi,<sup>1</sup> however, has failed to detect it in such urine.

*Coyote Urine.* The general data from the examination of the urine of this animal will be found in the last portion of Table II, p. 476. We here note a similar high specific gravity (1037–1049) to that observed in the case of the fox urine and an accompanying uniformity in creatinine-nitrogen output. Apart from these two features there are no important points in common between the general data from the examination of the urine of these two species of animal. In Table III are given the data on the *percentage distribution* of nitrogen as ammonia, creatinine and creatine. From a consideration of the data there tabulated we learn that the percentage of the total nitrogen output for the various days which was excreted in the form of ammonia varied from 3.2 per cent to 4.3 per cent. Placing both creatinine and creatine upon a similar basis it is seen that the percentage of the total nitrogen elimination which was present as creatinine-nitrogen ranged from 1.5 to 1.9 per cent whereas the limits for the creatine-nitrogen excretion are the somewhat similar ones of 1.4 and 2.2 per cent.

<sup>1</sup> Capaldi: *Zeitschr. f. physiol. Chem.*, xxiii, p. 87, 1897.

The *average* daily data from the analysis of coyote urine as contained in Table IV, p. 477, are rather more significant than the data discussed in the above paragraph. An examination of this table indicates that the average volume of urine voided per day upon an ingestion of 400 cc. of water was 217 cc. Thus the coyote excreted a volume of urine which was only slightly more than 50 per cent as great as the volume of water ingested. A similar relation was observed in the case of the fox urine (see p. 467). It is furthermore shown in this table that an average of 3.8 per cent of the total quantity of nitrogen excreted by this coyote was eliminated in the form of ammonia. The findings concerning the percentage output of creatinine and creatine are of particular interest. The creatinine nitrogen is seen to constitute 1.7 per cent of the total nitrogen, whereas 1.8 per cent of the total nitrogen was present as creatine. The presence of creatine-nitrogen in quantity equivalent to that of creatinine-nitrogen is not an unusual finding in the normal metabolism of dogs.

Calculated in terms of body weight units (kilograms) we find, as shown in Table V, p. 477, that 14.5 cc. of urine was excreted per kilogram of body weight and that this volume of urine had a total nitrogen value of 0.569 gram, an ammonia-nitrogen value of 0.022 gram whereas the excretion of creatinine-nitrogen was found to be 0.009 gram and that of creatine-nitrogen, 0.011 gram upon the same basis. Here again we observe the slight preponderance of creatine-nitrogen over creatinine-nitrogen.

The only analyses of coyote urine thus far reported, so far as we are aware are those of Swain.<sup>1</sup> A composite sample of urine obtained from a coyote fed 300 grams of meat and 50 grams of lard per day was found to contain 36.381 grams of total nitrogen per liter. Our total nitrogen figures calculated upon a similar basis are found to be equivalent to 39.43 grams of nitrogen per liter, thus showing rather close agreement with those of Swain. Data calculated thus can have but slight significance, however, inasmuch as they must undergo marked variations with the alterations of water ingestion. Swain found allantoin and kynurenic acid to be present in every sample of urine examined. He also demonstrated the presence of a crystalline substance "whose composition and

<sup>1</sup> Swain: *Amer. Journ. of Physiol.*, xiii, p. 30, 1905.



properties would go to show it to be some new constituent of carnivorous urine." This substance very nearly corresponded with Jaffé's<sup>1</sup> "Urocaninsäure" in composition and general properties. We have thus far made no effort to isolate the body described by Swain. However, Hunter<sup>2</sup> has recently failed to demonstrate the presence of this body in the urine of the coyote. He also failed to find any kynurenic acid.<sup>3</sup> We propose at some time in the near future to look for these substances in the urine of our coyote.

*Dog Urine.* The data obtained in this connection were those customarily obtained from normal dogs under such dietary conditions as were in force in our experiments. There is need for no discussion in this connection. The data are tabulated in Tables II, III, IV, and V, pp. 476 and 477 respectively.

#### COMPARISON OF THE DATA OBTAINED FROM THE ANALYSIS OF THE URINE OF THE FOX, DOG AND COYOTE.

This comparison will be limited to a consideration of the average daily data as tabulated in Table IV, p. 477 and of the data calculated upon the basis of the body weight of the animal as given in Table V, p. 477. The main point of interest in connection with the data included in Table IV is the percentage of the total nitrogen which was excreted as ammonia-nitrogen, creatinine-nitrogen and creatine-nitrogen by these animals of three different species. In the case of ammonia-nitrogen for example, we find that the coyote excreted 3.8 per cent of its total nitrogen in this form, whereas the fox showed the considerably lower value of 2.8 per cent. The ammonia-nitrogen content of the dog urine is higher than either of these values, the percentage, however (4.3 per cent), is rather closely comparable with that obtained from the analysis of the coyote urine. When we examine the creatinine-nitrogen data we again find the percentage of this form of nitrogen higher in the urine of the dog than in that of the fox or coyote. In this instance the fox urine yielded the intermediate value, whereas the coyote urine

<sup>1</sup> Jaffé: *Ber. d. deutsch. chem. Gesellschaft*, viii, p. 811, 1875.

<sup>2</sup> Hunter: private communication.

<sup>3</sup> The findings of Professor Hunter may be found upon another page of this number of this *Journal*.

showed the smallest output. The actual percentages as secured by analysis were 2.8 per cent for dog urine, 2.2 per cent for fox urine and 1.7 per cent for coyote urine. When we examine the data from the determination of creatine-nitrogen we find an interesting relation. It will be observed that the coyote urine and the dog urine possessed the same average percentage content of creatine-nitrogen, whereas the urine of the fox held only 50 per cent as much nitrogen in the form of creatine as did the urine of the other species. The actual percentage distribution of creatine-nitrogen was 1.8 per cent for the coyote and dog as against 0.9 per cent for the fox.

Probably our most significant data are summarized in Table V, p. 477. Here we have the data reduced to their lowest terms and expressed upon the "per kilogram of body weight" basis. Taking the data in order, as tabulated, we will first consider the urine volumes. It is noted that the fox excreted 10.9 cc. of urine per kilogram of body weight whereas the coyote excreted 14.5 cc. and the dog excreted 32.8 cc. calculated upon the same basis. Here we apparently have a rather remarkable relation when it is recalled that each animal was ingesting 26.6 cc. of water per kilogram of body weight. In other words the fox was excreting fluid equivalent to about 40 per cent of that ingested, whereas the coyote was excreting about 55 per cent and the dog was excreting over 120 per cent calculated upon the same basis. In connection with certain water-drinking tests the writer has shown<sup>1</sup> that the percentage of ingested fluid which is recovered in the urine ranges ordinarily from 30 to 70 per cent upon a moderate water ingestion, whereas the percentage of fluid excreted may exceed 90 per cent of that ingested under the influence of copious water drinking. However, we have never before obtained, over a period of six days, a volume of urine 20 per cent in excess of the volume of fluid ingested. Fortunately the dog used in the present study, was continued upon the same diet for a time after these comparative tests were finished, thus furnishing data on the urine volume for a period of fifteen days under normal conditions. By calculating the average daily urine volume for the entire period it is found to be 610 cc. This volume being excreted upon a daily ingestion of 700 cc. of water it is seen

<sup>1</sup> Hawk: *University of Pennsylvania Medical Bulletin*, xviii, p. 7, 1905.

that in the case of the dog the equivalent of 87 per cent of the ingested fluid was recovered in the urine. Even taking into consideration the entire fifteen days, however, we still find that the percentage of the ingested fluid which was accounted for in the urine volume was much greater than in the case of the fox and coyote. Bearing in mind the data obtained by us in connection with our water drinking studies it is apparent that the water ingestion calculated upon the basis of 26.6 cc. per kilogram of body weight was satisfactory for the metabolic uses of the fox and coyote organisms while at the same time it was apparently a *copious* water ingestion for the organism of the dog. We deduce this from the fact that the percentage excretion in the case of the fox and coyote, *i.e.*, 40 to 55 per cent, fell within the limits observed by us in moderate water-drinking (30 to 70 per cent) whereas in the case of the dog the percentage elimination of 87 per cent was well up in the scale of the percentages accompanying copious water ingestion.

Turning now to a consideration of the total nitrogen excretion and its distribution we observe in the first place that the dog excreted 0.66 gram of nitrogen per kilogram of body weight, whereas the coyote excreted 0.57 gram and the fox 0.44 gram. As we have already mentioned, each animal was ingesting 0.6 gram of nitrogen per kilogram of body weight. Therefore the indications are that the diet was abundant for the fox and coyote as shown by plus balances of 0.16 gram and 0.03 gram per kilogram of body weight whereas the balance in the case of the dog was a negative one of 0.06 gram. The feces were not subjected to daily analyses, but it was evident from such data as were obtained that the fecal nitrogen could in no way account for the variations in the balances just mentioned. Of course in this connection as well as in the other points of comparison we must not lose sight of the obvious fact that we have presented data from but a single individual of each species and that therefore the factor of individual characteristics may possibly account for some of the variations observed.

When we consider the forms in which the nitrogen occurred in the urine of the fox, dog and coyote we note that the relative quantities of the total nitrogen which appeared as ammonia-nitrogen were very similar for the coyote and dog, the actual figures being 0.027 gram for the dog as against 0.022 gram for the coyote. The fox urine yielded a value (0.012 gram), approximately only one-

half as great as those obtained from the analysis of the urine of the other species of animal. On the other hand, when we compare the data from the creatinine determinations it is seen that the weight of creatinine-nitrogen excreted per kilogram of body weight was very nearly the same for the fox as for the coyote this value being increased approximately 100 per cent in the case of the dog urine. The actual average daily excretions of creatinine-nitrogen for the coyote, fox and dog taken in order were 0.009 gram, 0.010 gram and 0.018 gram respectively.

The same general relation as that just discussed is noted when the data for the excretion of creatine-nitrogen are examined. That is, the content of this form of nitrogen in the urine of the dog was far above that present in the urine of either the fox or coyote. We fail to observe the uniformity in the data from the fox and coyote urine which was in force in the creatinine-nitrogen determinations. In the case of creatine the coyote excreted nearly four times as much nitrogen in this form per kilogram of body weight as did the fox, the exact figures being 0.011 gram for the coyote urine as against 0.003 gram for the fox urine. The dog excreted an average of 0.014 gram per kilogram of body weight.

The relation between creatinine-nitrogen and creatine-nitrogen is interesting. It will be observed for instance, that the coyote excreted slightly more of its nitrogen in the form of creatine than in the form of creatinine, whereas in the case of the dog the relation was reversed, the figures indicating a slightly higher value for the creatinine-nitrogen outgo. On the other hand, no such attempt at uniformity was observed in the case of the fox urine inasmuch as the data as tabulated indicate a ratio of creatinine-nitrogen to creatine-nitrogen of 3.3:1. When we calculate the combined output of creatinine-nitrogen and creatine-nitrogen for each animal we find that the coyote excreted 0.020 gram per kilogram of body weight whereas the dog excreted 0.032 gram upon the same basis. We thus see that the coyote's excretion was approximately two-thirds as great as that of the dog. If we follow the comparison further we find that the combined excretion for the fox was only 0.013 gram, which is a value only about two-thirds as great as that for the coyote and somewhat less than one-half as great as that for the dog.

When we take a general survey of the data calculated upon the "kilogram of body weight" basis, it is observed that from certain

standpoints the data from the fox and coyote present uniform findings, whereas from other standpoints the data obtained from the examination of the dog and coyote urine are more comparable. From no standpoint, however, are the data from the fox and dog equally comparable. In other words, taking the coyote data as the basis, we note, on the one side certain uniformities with the data from the fox figures, and on the other side certain uniformities with the dog data. For example when we consider the relation of the volume of the ingested fluid to the volume of the urine excreted we observe very similar findings for the fox and coyote, as compared with a far different finding for the dog. It will be recalled that about 40 per cent of the ingested fluid was recovered in the case of the fox, as against 55 per cent in the case of the coyote and 87 per cent in the case of the dog, provided a period of fifteen days was taken into account in the latter instance.

When we consider the relation between the ingested nitrogen and the total nitrogen output, we again observe that the conditions surrounding the fox and coyote data are very similar, each showing pronounced plus balance as against a minus balance for the dog. The creatinine-nitrogen data are also very similar in the case of the coyote and fox. The coyote and dog are rather closely uniform in the extent of their excretion of ammonia-nitrogen and creatinine-nitrogen. When we consider the close zoölogical relationship between the coyote (*Canis latrans*) and the dog (*Canis familiaris*) it is perhaps surprising that the urine of the two animals, fed the same ration per kilogram of body weight, does not yield data more uniformly comparable. From a similar viewpoint it might appear more less surprising that two animals with no closer zoölogical ties than unite the fox (*Vulpes fulvus*) and coyote (*Canis latrans*) should exhibit the uniformity noted in the reaction of their organisms to the same ration fed on the "per kilogram of body weight" basis.

In connection with the varied discussions of this paper we have not lost sight of the fact that the variations noted by us in the urinary data obtained from the analysis of the urine of the fox, dog and coyote, might perhaps be explainable solely upon the basis of individuality. We have demonstrated, for example, in certain experiments<sup>1</sup> that "two men of approximately the same age, weight

<sup>1</sup> Hawk: *Amer. Journ. of Physiol.*, x, p. 125, 1903.

and lung capacity, maintained upon the same constant diet and drinking precisely the same amounts of water and milk daily" may excrete widely different volumes of urine. In the case in point, the volumes were 1400 grams and 850 grams respectively. Similar conditions we know to surround the distribution of urinary nitrogen. Two normal persons of the same body weight and ingesting absolutely the same uniform diet may excrete widely different proportions of their total nitrogen output in the form of urea and ammonia for example. In fact we may go even further than this and say that the same individual ingesting a uniform diet will also exhibit rather pronounced variations in the nitrogen distribution at different times and for no apparent reason. In all metabolism studies therefore investigators cannot be too careful in drawing conclusions from slight variations in the distribution of urinary nitrogen.

#### SUMMARY.

A fox, dog and coyote were placed upon uniform diets apportioned upon the basis of 0.6 gram of nitrogen and 26.6 cc. of water per kilogram of body weight and the resultant urine subjected to examination. Determinations of total nitrogen, ammonia, creatinine and creatine were made. The fox and coyote exhibited pronounced plus balances, whereas the dog showed a minus balance, two observations which may be interpreted as indicating that 0.6 gram of nitrogen per kilogram of body weight was abundant for the fox and coyote but hardly sufficient for the dog.

The coyote and dog excreted very similar quantities of ammonia-nitrogen and creatine-nitrogen per kilogram of body weight, whereas the coyote excreted but 50 per cent as much creatinine-nitrogen as did the dog on the same basis.

The fox and coyote furnished very similar data as regards urine volume, total nitrogen output and creatinine-nitrogen excretion. As regards urine volume it was found that the fox excreted 10.9 cc. per kilogram of body weight, whereas the coyote excreted 14.5 cc. upon the same basis. The similar excretion in the case of the dog was 32.8 cc.

The creatinine-nitrogen data from the analysis of the urine of the fox and coyote show very close agreement, the fox excreting

0.010 gram and the coyote 0.009 gram per kilogram of body weight. The creatinine-nitrogen value for the dog urine was 100 per cent higher than these values.

The data from the analysis of the dog urine calculated upon the 'kilogram of body weight' basis were uniformly higher than those yielded by the urine of the fox or coyote. The similar data for the coyote were, with a single exception, higher than those obtained from the analysis of fox urine.

The dog and coyote each excreted approximately the same quantity of creatinine-nitrogen and creatine-nitrogen per kilogram of body weight. In the case of the fox the creatinine-nitrogen and creatine-nitrogen were present in the ratio 3.3:1.

The combined output of creatinine-nitrogen and creatine-nitrogen per kilogram of body weight in the case of the coyote was approximately only two-thirds as great as that of the dog, whereas that of the fox was less than 50 per cent as great as that of the dog.

Calculated upon the basis of body weight units (kg.) there were no points of similarity between the data obtained from the analysis of the fox and dog urine.

The samples of the coyote and fox urine were of a uniformly higher specific gravity than those of the dog urine.

No search was made for "Urocaninsäure" in the urine of the coyote.

TABLE I.

*Daily Diet of Fox, Dog and Coyote.*

CONSTITUENTS OF DIET	NITROGEN CONTENT	AMOUNT FED		
		Fox	Dog	Coyote
	<i>per cent.</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>
Meat .....	3.618	71	400	228
Crackers.....	1.318	18	100	57
Lard.....	0.010	8	45	26
Bone Ash.....	0.010	4*	12	10*
Water.....		125	700	400

\*Extra bone ash added to diet in order to insure feces of proper consistency for handling.

TABLE II.

*Examination of Fox, Dog and Coyote Urine.*

DAY OF EXPT.	VOLUME OF URINE CC.	SPECIFIC GRAVITY	TOTAL NITROGEN grams	AMMONIA NITROGEN grams	CREATININE NITROGEN grams	CREATINE NITROGEN grams
<i>Fox Urine.</i>						
1	65	1040	1.981	0.076	0.050	0.035
2	56	1044	2.154	0.037	0.050	0.023
3	47	1045	1.997	0.062	0.040	0.006
4	52	1044	2.133	0.060	0.050	0.006
5	45	1045	2.116	0.052	0.040	0.001
6	43	1043	2.078	0.056	0.040	0.004
<i>Dog Urine.</i>						
1	963	1026	17.360	0.608	0.421	0.470
2	448	1018	6.050	0.390	0.187	—
3	1330	1026	24.970	0.948	0.666	0.484
4	360	1031	8.920	0.330	0.311	0.108
5	1028	1028	23.192	1.000	0.622	0.331
6	1043	1028	23.960	0.986	0.608	0.430
<i>Coyote Urine.</i>						
1	155	1048	7.702	0.334	0.130	0.150
2	153	1049	7.764	0.251	0.130	0.120
3	166	1046	7.693	0.256	0.130	0.110
4	282	1037	9.075	0.368	0.170	0.150
5	200	1044	9.258	0.390	0.140	0.190
6	347	1029	9.848	0.358	0.150	0.220



TABLE III.

Percentage Distribution of Nitrogen as Ammonia, Creatinine and Creatine.

DAY OF EXPERI- MENT	SPECIES OF ANIMAL								
	FOX			DOG			COYOTE		
	Ammonia N.	Creatinine N.	Creatine N.	Ammonia N.	Creatinine N.	Creatine N.	Ammonia N.	Creatinine N.	Creatine N.
1	3.8	2.5	1.8	3.5	2.4	2.7	4.3	1.7	1.9
2	1.7	2.3	1.1	6.4	3.1	—	3.2	1.7	1.6
3	3.1	2.0	0.3	3.8	2.7	1.9	3.3	1.7	1.4
4	2.8	2.3	0.3	3.7	3.5	1.2	4.1	1.9	1.7
5	2.5	1.9	0.1	4.3	2.7	1.4	4.2	1.5	2.1
6	2.7	2.0	1.9	4.1	2.5	1.8	3.6	1.5	2.2

TABLE IV.

Average Daily Data from Analysis of Fox, Dog and Coyote Urine.

SPECIES OF ANIMAL	AVERAGE EXCRETION PER DAY							
	URINE VOLUME	Grams.				Per Cent.		
		Total N.	Ammonia N.	Creatinine N.	Creatine N.	Ammonia N.	Creatinine N.	Creatine N.
Fox.....	51	2.076	0.057	0.045	0.013	2.8	2.2	0.9
Dog.....	862	17.409	0.710	0.469	0.365	4.3	2.8	1.8
Coyote.....	217	8.557	0.326	0.140	0.160	3.8	1.7	1.8

TABLE V.

Average Daily Data per Kilogram Body Weight.

SPECIES OF ANIMAL	URINE VOLUME	TOTAL N.	AMMONIA N.	CREATININE N.	CREATINE N.
	cc.	grams.	grams.	grams.	grams.
Fox.....	10.9	0.442	0.012	0.010	0.003
Dog.....	32.8	0.662	0.027	0.018	0.014
Coyote .....	14.5	0.569	0.022	0.009	0.011



# THE FERMENTATION OF CITRIC ACID IN MILK.

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Two years ago while testing the method of Beau<sup>1</sup> for the estimation of citric acid in milk we made the observation that milk which, when fresh, had contained 0.21 per cent of citric acid, when soured contained none.

It is known from the work of Macagno<sup>2</sup> that citric acid may be broken down by bacteria with the formation of acetic and propionic acids. Hydrogen, carbon dioxide, alcohol, butyric and succinic acids have, in addition, been found to arise from the bacterial decomposition of citrates.<sup>3</sup>

In confirmation of our first observation the following experiments were made.

*Experiment 1.* A sample of perfectly fresh milk was obtained and its citric acid content determined at varying intervals after inoculating with "butter starter." The results were as follows:

Age in Hours.	Acidity, Cc. of $\frac{N}{10}$ Alkali to Neutralize 100 cc.	Citric Acid, Per Cent.
4	26.4	0.224
15	40.0	0.224
28	curdled	0.224
35	curdled	0.217
50	curdled	0.053
60	curdled	0

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<sup>1</sup> *Rev. gen. lait.*, iii, p. 385.

<sup>2</sup> *Gaz. chem. ital.*, xi, p. 443, cited from Thorpe's *Dictionary of Applied Chemistry*.

<sup>3</sup> *Beilsteins Handbuch. d. org. Chem.*

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*Experiment 2.* A second sample of milk treated as in (1) gave the following:

Age in Hours.	Acidity.	Citric Acid.
12	20.0 cc.	0.199
36	84.0 cc.	0.072
60	curdled	0

The whey obtained at the end of experiment 2 was partly neutralized with sodium hydroxide and calcium citrate added. Immediate analysis showed 0.119 per cent of citric acid; analysis after standing twenty-four hours showed none.

The following experiment shows that citric acid may be decomposed by fermentation in lactose-bouillon.

To each of four flasks containing 500 cc. of bouillon with 1 per cent of lactose were added 5 grams of calcium citrate. After sterilization, two were inoculated with 1 cc. of buttermilk. In these two, at the end of the twelfth day, the insoluble calcium citrate had disappeared: in the controls it remained undissolved. Chemical analysis of the contents of the inoculated flasks revealed no citric acid: on distillation with steam after acidification with sulphuric acid, a large amount of acetic acid was recovered, identified by the formation of acet-p-toluide, m.p., 148.°

An experiment was made which showed that citric acid may be a source of some of the volatile acids which are found in sour milk.

A sample of fresh milk was divided into two portions. To one was added calcium citrate. Both were allowed to stand for fourteen days. At the end of this time, neither contained any citric acid. One-hundred cc. of the whey from the portion to which citrate had been added yielded on distillation volatile acid equivalent to 45.7 cc.  $\frac{N}{16}$  acid; 100 cc. of whey from the portion to which no citrate had been added yielded the equivalent of 32.3 cc. of  $\frac{N}{16}$  volatile acid. The acid was shown to be acetic by formation of acet-p-toluide.

To determine whether pure citrate, in the absence of the other constituents of milk, may be the source of the acetic acid formed in souring and to study the action of pure cultures of common dairy bacteria upon citrates, the following medium was prepared.

Acid sodium phosphate.....	5.0 grams.
Magnesium sulphate.....	5.0 grams.
Potassium chloride.....	0.5 gram.
Official ammonium citrate solution <sup>1</sup> .....	50.0 cc.
Water, to make.....	1000.0 cc.

In a preliminary test it was found that eight days after the inoculation of the above solution with sour milk no citric acid could be detected and that carbon dioxide and acetic acid had been formed.

Portions of this medium were inoculated with pure cultures of various bacteria, allowed to stand twenty days, then tested for citrate. The citrate disappeared completely under the influence of *Bact. lactis aerogenes*: no effect upon the citrate was produced by *Bact. lactis acidi* (three strains), *Bact. lactis aureum II* (two strains), *Strept. lacticus* (three strains), *Strept. lactic citreus* or *Micrococcus lactis varians* (two strains).<sup>2</sup> A quantitative estimation of the volatile acid formed from the citrate by *Bact. lactis aerogenes* was made. Five-hundred cc. of the fluid were distilled with steam after acidification with sulphuric acid. The acidity of the distillate was equivalent in one case to 40.8 cc. of normal acid, in another to 44 cc. Acetic acid was identified by the formation of acet-p-toluide, m. p., 148°. Of the original culture medium, 500 cc. contained ammonium citrate in amount equivalent to 4.2285 grams of citric acid. If one molecule of citric acid is capable of decomposing into two molecules of acetic the theoretical yield of acetic acid would be 2.6428 grams. The total acidity of the distillate calculated as acetic acid amounted in one case to 2.448, in the other to 2.64 grams of acetic acid.

Since *Bact. lactis acidi* is the predominant organism in buttermilk and in "butter starter" it seemed strange that the organism in pure culture did not break down the citric acid of our medium.

<sup>1</sup> Cf. *Bulletin 107* (revised), Bureau of Chemistry, U. S. Dept. of Agriculture, p. 1. Fifty cc. of this solution contains the equivalent of 8.457 grams of citric acid.

<sup>2</sup> All of these cultures were of organisms isolated from Cheddar cheese. They are classified according to Conn, Esten and Stocking: "Classification of Dairy Bacteria," *Annual Report*, Storrs Agricultural Experiment Station, 1906.

An experiment was made which showed that this bacterium, added in pure culture to milk containing ammonium or calcium citrate, did not exert any action upon it. On the other hand, ammonium or calcium citrate in milk inoculated either with pure culture of *Bact. lactis aerogenes* or with a sample of the buttermilk from which the *Bact. lactis acidi* had been isolated was completely decomposed. Obviously the disappearance of citric acid in souring milk is not due to *Bact. lactis acidi*.

In the process of cheese making, the citric acid of the milk disappears before the cheese is put in the press. This is shown by the following data.

	Citric Acid. Gm.
Fresh milk contained.....	0.203
Whey when drawn contained.....	0.118
First whey from press contained.....	0.000
Curd when whey was drawn contained.....	trace
Curd when put in press contained.....	0.000

From these experiments the following conclusions may be drawn:

1. During the souring of milk, the citric acid contained in it is changed into acetic acid and carbon dioxide.
2. Of the common dairy bacteria tested the only one found to have the power of decomposing citric acid was *Bact. lactis aerogenes*.
3. Citric acid when fermented by *Bact. lactis aerogenes* yields two molecules of acetic acid for every one of citric acid.
4. In the process of cheese-making, citric acid of milk is entirely fermented before the curd is pressed.

# THE DETERMINATION OF INORGANIC AND ORGANIC PHOSPHORUS IN MEATS.

BY H. S. GRINDLEY AND E. L. ROSS.

*(From the Laboratory of Physiological Chemistry, Department of Animal Husbandry, University of Illinois. Nutrition Investigations. Publication No. 29.)*

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## INTRODUCTION.

In 1906 Emmett and Grindley<sup>1</sup> concluded from their investigations, that the Hart and Andrews<sup>2</sup> method for the determination of inorganic and organic phosphorus in feeding stuffs, could not be used directly for determining these different forms of phosphorus in aqueous extracts of flesh, on account of the large quantity of soluble proteins present. They concluded, however, that if the soluble proteins were removed by coagulation, then the neutral ammonium molybdate method proposed by Hart and Andrews could be used for the separation and estimation of inorganic and organic phosphorus. Investigations in this laboratory since that time have clearly tended to confirm the accuracy of the method proposed and have also confirmed the conclusion of Emmett and Grindley that a considerable proportion of the soluble phosphorus of beef flesh, *i.e.*, from 50 to 90 per cent, occurs in the form of inorganic phosphorus.

In the meantime Forbes and associates<sup>3</sup> have made extensive and thorough investigations of the methods for the quantitative determination of inorganic and organic phosphorus in vegetable and animal substances. Their work clearly demonstrated the accuracy of the Emmett and Grindley method for the determin-

<sup>1</sup> *Journ. Amer. Chem. Soc.*, xxviii, p. 26, 1906.

<sup>2</sup> *Amer. Chem. Journ.*, xxx, p. 470, 1903.

<sup>3</sup> *Ohio Agr. Exp. Sta. Bull.*, 215, p. 484, 1910.

ation of these forms of phosphorus in animal substances, not only by direct test but also by comparison with two other entirely different methods, which they proposed, *i.e.*, the magnesia mixture and the acid-alcohol methods.

Still later Trowbridge and Stanley,<sup>1</sup> and Francis and Trowbridge<sup>2</sup> have published results which, as they maintain, prove that the Emmett and Grindley method does not give high enough results for organic phosphorus in cold water extracts of flesh, for the reason that the heat of coagulation involved in this method changes nearly all of the organic phosphorus to the inorganic form. They also maintain that their researches show that there is a progressive splitting up of the organic phosphorus compounds in beef flesh during the process of cooking so that in well-done meats practically all of the phosphorus is present in the inorganic form. Trowbridge and associates used the method that Siegfried and Singewald<sup>3</sup> employed in separating inorganic and organic phosphorus in meat extracts, *i.e.*, the precipitation of the inorganic phosphorus by a solution of barium chloride in the presence of an excess of ammonium hydroxide.

The results obtained by Trowbridge and associates were so strikingly different from those obtained in this laboratory and those reported by Forbes and associates that we were led to make a somewhat exhaustive and careful comparative study of the various methods that have been proposed and used for the separation and estimation of inorganic and organic phosphorus in meats.

#### METHODS USED.

In this paper we report the results of a comparative study of the magnesia-mixture method of Forbes and associates, the neutral ammonium molybdate method of Emmett and Grindley, and the barium precipitation method of Siegfried and Singewald, which was first proposed by Trowbridge and associates for the direct determination of inorganic phosphates in fresh meats.

<sup>1</sup> *Journ. Ind. and Eng. Chem.*, ii, p. 212, 1910.

<sup>2</sup> *This Journal*, vii, pp. 481-501, 1910; and viii, pp. 81-93, 1910.

<sup>3</sup> *Zeitschr. f. Nähr. Genussm.*, x, p. 521, 1905.



Briefly stated, the procedure in each of these methods was as follows:

**FORBES MAGNESIA-MIXTURE METHOD.** To each of three portions of 100 cc. of the water extracts of beef, 20 cc. of magnesia-mixture were added from a burette at the rate of about one drop per second, with constant stirring. After standing 15 minutes, 20 cc. of ammonia water, of specific gravity 0.90, were added and the solutions were allowed to stand for at least 12 hours. They were then filtered through double quantitative filters, the precipitates being washed with 2.5 per cent ammonia water. The upper filter containing the precipitate, after being transferred to the beaker in which the precipitation was effected, was digested with about 35 cc. of dilute nitric acid. The acid solution was then filtered through the second filter which had been left undisturbed in the funnel, the beakers and filters being thoroughly washed with hot water. To each of the filtrates about 15 cc. of ammonia water of specific gravity 0.90, were added. The solutions were then very slightly acidified with pure nitric acid, litmus paper being used as an indicator, diluted to approximately 125 cc., and then warmed to 60°, the phosphorus determination being continued from here on as usual.

**EMMETT-GRINDLEY METHOD.** Three portions of 100 cc. each of the water extract of beef were evaporated to approximately 25 cc. While hot, the solutions were filtered, the beakers, coagulated protein, and filters being thoroughly washed with hot water. To the filtrates, measuring about 125 cc., 10 grams of ammonium nitrate were added. They were then heated to 60° and from 5 to 8 cc. of nitric acid of specific gravity 1.20, and 100 cc. of a clear, neutral ammonium molybdate solution added to each. The temperature of the solutions was then again brought to 60° and maintained at this point for 15 minutes, with vigorous stirring every few minutes. The solutions were then removed from the steam bath and allowed to stand for at least 2 hours before filtration. The beakers, precipitates, and filters were thoroughly washed with a slightly acidified solution of ammonium nitrate. The yellow precipitate upon the filter and in the beaker was dissolved in dilute ammonium hydroxide (2.5 per cent solution) and hot water. The ammoniacal solutions were neutralized with nitric acid and diluted to a volume of about 150 cc. After the addition of 5 grams of ammonium nitrate the solutions were heated upon the steam bath to 60° and then while stirring, 5 cc. of concentrated nitric acid and 25 cc. of clear acid molybdic solution were added. The solutions were maintained at a temperature of 60° for 15 minutes. From here on the phosphorus determination was continued as usual.

**SIEGFRIED AND SINGEWALD METHOD.** To each of three portions of 100 cc. of the water extracts of beef 50 cc. of a 10 per cent barium chloride solution and 10 cc. of a 10 per cent solution of ammonium hydroxide were added. The solutions were stirred every 15 minutes for a period of one hour, allowed to stand undisturbed for at least 12 hours, and then filtered through double quantitative filters. The beakers, precipitates, and filters were repeatedly

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washed with small quantities of wash water containing 10 cc. of the barium chloride solution and 10 cc. of the ammonium hydroxide solution per liter. The upper filters containing the precipitates were placed in the beakers in which the precipitation occurred, and digested with about 35 cc. of dilute nitric acid. The acid solution was then filtered through the second filter which had not been removed from the funnel, the beakers and filters being thoroughly washed with hot water. To each of the filtrates about 15 cc. of ammonia water of specific gravity 0.90, were added and each was then very slightly acidified with pure nitric acid, litmus paper being used as an indicator. The solutions were then diluted to approximately 125 cc. and warmed to 60°, the phosphorus determination being continued from here on as usual.

In studying the method of Siegfried and Singewald in this laboratory, four years ago, Grindley and Petersen<sup>1</sup> used a 10 per cent solution of barium nitrate and a 10 per cent solution of ammonium hydroxide as the precipitant for phosphoric acid. In this earlier work it was clearly proven that these reagents precipitated the same amount of phosphorus from an aqueous solution of potassium dihydrogen phosphate as did the ammonium molybdate solution. It was also demonstrated that inorganic phosphorus is completely precipitated by barium nitrate in solutions made alkaline with barium hydroxide, and also by barium hydroxide alone. As a barium nitrate solution had been used in our earlier work, its use was continued at first in connection with the present study.

The results of the tests are given in the following four tables.

<sup>1</sup> Unpublished manuscript.

TABLE I.

DETERMINATION OF INORGANIC PHOSPHORUS IN AQUEOUS EXTRACTS OF  
RAW BEEF BY VARIOUS METHODS.*Results expressed in per cent of phosphorus.*

METHODS USED IN THE DETERMINATIONS.	FIRST DETERMINATION.	SECOND DETERMINATION.	THIRD DETERMINATION.	AVERAGE.	INORGANIC IN PER CENT OF TOTAL PHOSPHORUS.
<i>Beef Rib.</i>					
Total phosphorus.....	0.116	0.118	0.114	0.116	....
Emmett and Grindley.....	0.108	0.108	0.108	0.108	93.1
Forbes magnesia mixture.....	0.101	0.105	0.103	0.103	89.7
25 cc. of barium nitrate.....	0.103	0.103	0.102	0.103	89.7
<i>First Sample of Beef Round</i>					
Total phosphorus.....	0.137	0.137	.....	0.137	....
Emmett and Grindley.....	0.106	0.107	0.106	0.106	77.4
Forbes magnesia mixture.....	0.104	0.103	0.105	0.104	75.9
25 cc. of barium nitrate.....	0.104	0.104	0.105	0.104	75.9
<i>Second Sample of Beef Round</i>					
Total phosphorus.....	0.142*	0.154	0.157	0.155	....
Emmett and Grindley.....	0.131	0.131	0.132	0.131	84.5
Forbes magnesia mixture.....	0.134	0.134	0.131	0.133	85.7
25 cc. of barium nitrate.....	0.132	0.131	0.130	0.131	84.5

\*Not included in the average.

TABLE II.

DETERMINATION OF INORGANIC PHOSPHORUS IN AQUEOUS EXTRACTS OF  
RAW BEEF BY VARIOUS METHODS.*Results expressed in per cent of phosphorus.*

METHODS USED IN THE DETERMINATIONS.	FIRST DETERMINATION.	SECOND DETERMINATION.	THIRD DETERMINATION.	AVERAGE.	INORGANIC IN PER CENT OF TOTAL PHOSPHORUS
<i>First Sample of Beef Chuck.</i>					
Total phosphorus.....		0.128	0.127	0.127	
Forbes magnesia mixture.....	0.097	0.095	0.095	0.096	75.6
25 cc. of barium nitrate. Dilution					
100 cc.....	0.095	0.092	0.094	0.094	74.0
25 cc. of barium nitrate. Dilution					
500 cc.....	0.093	0.093	0.092	0.093	73.2
<i>Second Sample of Beef Chuck.</i>					
Total phosphorus.....	0.156	0.157	0.158	0.157	
25 cc. Ba(NO <sub>3</sub> ) <sub>2</sub> . Not coagulated.					
Dilution 100 cc.....	0.085	0.084	0.084	0.084	53.2
25 cc. Ba(NO <sub>3</sub> ) <sub>2</sub> . Not coagulated.					
Dilution 500 cc.....	0.083	0.083	0.082	0.083	52.9
25 cc. Ba(NO <sub>3</sub> ) <sub>2</sub> . Coagulated. Dilu-					
tion 100 cc.....	0.087	0.087	0.087	0.087	55.4
25 cc. Ba(NO <sub>3</sub> ) <sub>2</sub> . Coagulated. Di-					
lution 500 cc.....	0.084	0.084	0.084	0.084	53.2

TABLE III.

## DETERMINATION OF PHOSPHORUS IN THE AQUEOUS EXTRACTS OF RAW MEATS BY VARIOUS METHODS.

*Results expressed in per cent of phosphorus.*

METHODS USED IN THE DETERMINATIONS.	FIRST DETERMINATION.	SECOND DETERMINATION.	THIRD DETERMINATION.	AVERAGE.	INORGANIC IN PER CENT OF TOTAL PHOSPHORUS.
<i>Beef Round.</i>					
Total phosphorus . . . . .	0.149	0.149	0.149	0.149	..
Emmett and Grindley . . . . .	0.101	0.110	0.105	0.105	70.5
Magnesia mixture, before coagulation. . . . .	0.109	0.108	0.110	0.109	73.2
Magnesia mixture, after coagulation . . . . .	0.106	0.109	0.107	0.107	71.8
10 cc. of BaCl <sub>2</sub> solution . . . . .	0.014	0.013	0.012	0.013	8.7
25 cc. of BaCl <sub>2</sub> solution . . . . .	0.090	0.098	0.098	0.095	63.8
50 cc. of BaCl <sub>2</sub> solution . . . . .	0.102	0.105	0.106	0.104	69.8
<i>Beef Shank.</i>					
Total phosphorus . . . . .	0.125	0.123	0.126	0.125	....
Emmett and Grindley . . . . .	0.097	0.099	0.098	0.097	77.6
Magnesia mixture, after coagulation . . . . .	0.098	0.098	0.097	0.098	78.4
5 cc. of BaCl <sub>2</sub> solution . . . . .	0.020	0.018	.....	0.019	15.2
10 cc. of BaCl <sub>2</sub> solution . . . . .	0.024	0.020	0.024	0.023	18.4
25 cc. of BaCl <sub>2</sub> solution . . . . .	0.076	0.082	0.076	0.078	62.4
Trowbridge, using 5 cc. of BaCl <sub>2</sub> * . . . . .	0.044	0.042	0.046	0.044	35.2
Trowbridge, using 25 cc. of BaCl <sub>2</sub> . . . . .	0.099	0.100	0.103	0.101	80.8

\*Trowbridge and associates determined the phosphorus in the filtrate from the barium phosphate precipitate. In all our work except these two determinations we determined the phosphorus directly in the precipitate produced by the barium salt.

TABLE IV.  
 INORGANIC PHOSPHORUS IN AQUEOUS EXTRACTS OF BEEF ROUND.  
*Results expressed in per cent of phosphorus.*

METHODS USED IN THE DETERMINATIONS	FIRST DETERMINATION.	SECOND DETERMINATION.	THIRD DETERMINATION.	AVERAGE.	INORGANIC IN PER CENT OF TOTAL PHOSPHORUS.
<i>Raw Beef Round Before Removal of the Coagulated Protein by Heat</i>					
Total phosphorus.....	0.154	0.155	.....	0.154	....
5 cc. of BaCl <sub>2</sub> solution.....	0.013	0.012	0.013	0.013	8.4
10 cc. of BaCl <sub>2</sub> solution.....	0.015	0.012	0.015	0.014	9.1
25 cc. of BaCl <sub>2</sub> solution.....	0.085	0.091	0.082	0.086	55.8
50 cc. of BaCl <sub>2</sub> solution.....	0.086	0.086	.....	0.086	55.8
<i>Same Beef Round After Complete Removal of the Coagulable Protein by Heat.</i>					
5 cc. of BaCl <sub>2</sub> solution.....	0.078	0.074	0.073	0.075	48.7
10 cc. of BaCl <sub>2</sub> solution.....	0.081	0.084	0.081	0.082	53.2
25 cc. of BaCl <sub>2</sub> solution.....	0.083	0.082	.....	0.083	53.9
50 cc. of BaCl <sub>2</sub> solution.....	0.083	0.085	.....	0.084	54.5

## DISCUSSION OF THE RESULTS.

It is apparent from the results given in Table I, that in two out of the three samples of beef analyzed, the Emmett and Grindley method gave slightly higher results for inorganic phosphorus than did the Forbes magnesia-mixture method, or the Siegfried and Singewald method using barium nitrate as the precipitant, while in the other sample, the first method gave slightly lower results than the magnesia-mixture method, but approximately the same results as the barium nitrate method. It is quite evident that these slight differences could not possibly account for the results obtained and the conclusions deduced by Trowbridge and associates.

It was then thought that the rather high dilution of the solutions used in the experiments of the latter investigators may have led to the remarkable results which they have reported. The data given in Table II, however, do not confirm this supposition, for it is apparent that 25 cc. of a 10 per cent solution of barium nitrate in the presence of 10 cc. of a 10 per cent solution of ammonium hydroxide, precipitates practically as much phosphorus from a volume measuring 100 cc. as from a volume measuring 500 cc. In addition the results of this table confirm those given in Table I, in that the magnesia-mixture method and the barium nitrate method as here used, give practically the same results for inorganic phosphorus in cold water extracts of beef.

It was then thought that the volume of barium chloride solution used in the work of Trowbridge and associates was not sufficient to bring about complete precipitation in the presence of soluble proteins of the cold water extracts of flesh. That this supposition was correct seems quite evident from the results given in Table III. In the first place the quantity of phosphorus precipitated from cold water extracts of beef increases as the volume of the barium chloride solution used for precipitation is increased from 5 to 25 or 50 cc. For example, in the case of the sample of beef shank 5, 10, and 25 cc. of barium chloride solution gave 0.019, 0.023, and 0.078 per cent of inorganic phosphorus, respectively, such data indicating that 15.2, 18.4, and 62.4 per cent of the total soluble phosphorus of fresh beef shank existed in the inorganic form. In the sample of beef round 10, 25, and 50 cc. of barium

chloride solution gave 0.013, 0.095, and 0.104 per cent of phosphorus, respectively, such results pointing to the conclusion that 8.7, 63.8, and 69.8 per cent of the total soluble phosphorus was present in the inorganic form.

In the second place, the inorganic phosphorus obtained by the magnesia-mixture method before coagulation and after coagulation of the proteins in the same water extract of beef round, varied but slightly, the former being 0.109 per cent and the latter 0.107 per cent, the former result would indicate that 73.2 per cent of the total soluble phosphorus existed in the inorganic form and the latter 71.8 per cent. In the third place the results obtained by the Emmett and Grindley method, which requires coagulation of the soluble protein, compares quite closely with the results obtained by the Forbes method, either with or without coagulation.

The above mentioned facts are, in close agreement with the data given in Table IV. In the sample of raw beef round before removal of the coagulated protein by heat, 5, 10, 25, and 50 cc. of barium chloride solution gave 0.013, 0.014, 0.086, and 0.086 per cent of inorganic phosphorus, respectively, equivalent to 8.4, 9.1, 55.8 and 55.8 per cent of the total soluble phosphorus. In the same water extract of the same beef round after complete removal of the coagulable protein by heat 5, 10, 25, and 50 cc. of barium chloride solution gave 0.075, 0.082, 0.083, and 0.084 per cent of phosphorus, respectively, equivalent to 48.7, 53.2, 53.9, and 54.5 per cent of the total soluble phosphorus. Evidently coagulation did not change organic phosphorus to the inorganic form, but the soluble coagulable protein present in the uncoagulated cold water extract prevented the complete precipitation of the inorganic phosphorus when only 5 or 10 cc. of the barium chloride solution was used in the precipitation. The addition of 25 or 50 cc. of the barium chloride solution completely precipitated the inorganic phosphorus, even in the presence of the soluble coagulable protein. Furthermore these volumes of the precipitant do not precipitate any more phosphorus after coagulation than before. On the other hand 5 and 10 cc. portions of the barium chloride solution did precipitate much more phosphorus after coagulation than they did in the same solutions before coagulation, thus demonstrating clearly that the soluble protein hindered complete precipitation by these smaller volumes of the precipitant. Since Trowbridge



and associates used only 5 cc. of a 10 per cent solution of barium chloride, it is quite apparent that the remarkably low and variable results which they obtained for inorganic phosphorus in uncooked meats were in the main due to the use of an insufficient quantity of the barium chloride solution. The apparent change of organic phosphorus to the inorganic form which they found upon coagulation of aqueous extracts of flesh was evidently due to the same causes.

#### CONCLUSIONS.

1. The results here presented seem to indicate clearly that the neutral ammonium molybdate method as modified by Emmett and Grindley, the magnesia-mixture method, as proposed by Forbes and associates, and the barium chloride method of Siegfried and Singewald, when used with proper precautions, give practically the same results for inorganic phosphorus in cold water extracts of beef.

2. Judging from the data here presented it is evident that the coagulation of the protein of the aqueous extracts of flesh by heat does not change organic phosphorus to the inorganic form to any appreciable extent.

Further study of the methods for the separation and estimation of inorganic and organic phosphorus in raw and cooked flesh and meat products is now being made in this laboratory.



## **THE PERSISTENCE OF STRYCHNINE IN A CORPSE.**

By MARSHALL P. CRAM AND PHILIP W. MESERVE.

(Received for publication, November 7, 1910.)

Certain conditions under which strychnine has been found in an exhumed body considerable time after death seem worthy of record.

On November 15, 1909, a man fifty-three years old died under suspicious circumstances. The body was buried on November 19, 1909, but on November 29 of the same year was disinterred, certain organs removed and sent to the late Franklin C. Robinson for analysis. Prof. Robinson's notes record that he found strychnine, but, owing to illness, he was unable to appear personally at the trial. The body, therefore, was exhumed a second time on March 18, 1910, and other organs removed which were sent to the senior author. The body had been frozen most of the time but the grave when opened was full of water which was allowed to drain off. An embalming fluid of acid reaction had been used when the body was first buried, which made it appear likely that any strychnine would be dissolved out.

The method used for determining strychnine was the usual one of extracting with alcohol made acid with acetic acid, evaporating the extract to soft dryness, extracting the residue with water, extracting the acid water solution with chloroform, and then the same solution made alkaline with chloroform, the strychnine coming out in the last chloroform extract. The strychnine was then purified by means of absolute alcohol.

From 454 grams of the lung, 133 grams of kidney, 446 grams of muscle, 850 grams of small intestine and 560 grams of brain, each being tested separately, no strychnine was obtained. From 803 grams of liver was obtained 0.0015 gram of strychnine, and from the spinal cord 0.0033 gram. The spinal cord itself weighed 25 grams.

The note books of Prof. Robinson record that in 290.0 grams of stomach contents he found 0.0277 gram of strychnine, and in 45 grams of kidney 0.0003 gram of strychnine.

The spinal cord, being better protected from the action of the water in the grave, which was acid from the embalming fluid, had retained the strychnine better than the other parts.

## NOTE ON THE DETERMINATION OF AMMONIA IN URINE.

By OTTO FOLIN.

(From the Biochemical Laboratory of the Harvard Medical School, Boston.)

(Received for publication, November 24, 1910.)

The inadequacy of sodium carbonate for the decomposition of ammonium magnesium phosphate, as repeatedly described by Steel<sup>1</sup>, is a fact that is well worth knowing in connection with the determination of ammonia in urine. This phosphate is, however, seldom if ever found in human urines which are not decomposed or alkaline, and when present is found during the microscopic examination. The possible occurrence of minute amounts of such phosphates hardly warrants the substitution of sodium hydrate and sodium chloride for sodium carbonate and sodium chloride<sup>2</sup> in all ammonia determinations as recommended by Steel.<sup>3</sup> There can scarcely be any doubt but that the carbonate is the safer reagent, and therefore to be preferred unless weightier reasons can be found against it than the possible occurrence of traces of the triple phosphate.

<sup>1</sup> Steel: This *Journal* vii, p. 365, 1910; vii, p. lviii; v, p. 85. *Proc. of the Soc. for Exp. Biol. and Med.*, vi, p. 127, 1909.

<sup>2</sup> Steel's statement (this *Journal*, vii, p. 371) that I have abandoned the use of sodium chloride in connection with ammonia determinations is not quite correct. When I have available an effective air stream capable of removing the ammonia from 20 cc. in about an hour, I rarely use it with urine, but whenever the air current is not so strong, or when the amount of liquid is considerably more than 20 cc., or whenever I fear the presence of unstable nitrogenous products, I still use it.

Because of the phosphates a mixture of potassium oxalate and sodium chloride (1 : 1) or potassium oxalate alone is probably preferable to sodium chloride.

<sup>3</sup> The use of sodium hydrate in connection with the air current method for determining ammonia in urine was advocated by Moritz several years ago, but it appears to have failed to meet with much approval: *Archiv für klinische Medizin*, lxxxiii, p. 567, 1905.

Ammonia determinations in urines containing excessive sediments of triple phosphates are of course at best not perfectly satisfactory unless the conditions warrant a preliminary solution of the entire sediment by the addition of acid. After the phosphate has been dissolved the ammonia can be determined in the usual manner with sodium carbonate provided that the re-formation of the phosphate is prevented. This is accomplished by the addition of an excess of potassium oxalate (7-10 grams to 25 cc. of urine).

## THE DETERMINATION OF TOTAL SULPHUR IN URINE.

By STANLEY R. BENEDICT.

(From the Laboratory of Chemical Pathology, Cornell Medical School, New York.)

(Received for publication, December 3, 1910.)

In a recent number of this *Journal* Denis<sup>1</sup> has published a paper dealing with a method proposed by the present writer about a year ago for the estimation of total sulphur in urine,<sup>2</sup> and proposes a modification of the process which is said to yield satisfactory results. There are certain aspects of this communication offered by Denis upon which it may be permissible to offer a few comments. Denis states that he made forty attempts to make use of the present writer's process, every one of which resulted in "utter failure." It is to be regretted that Denis offers not so much as one single set of figures as a basis for his wholesale condemnation of the original process. The possible implication contained in Denis' paper that the present writer offered his method without adequate preliminary testing is not correct. Prior to its publication this method had been worked with continuously for nearly a year, during which time over four hundred urinary analyses were made in duplicate by the process, most of these determinations having been carried out in the laboratory of Professor Long in Chicago, by three analysts other than the present writer. In all these analyses there were less than 2 per cent of repeats, from any cause. For the most part these facts were mentioned in my original paper on the subject, and furthermore, analyses were reported by the copper nitrate method and by Folin's peroxide method, showing excellent agreement. Denis dismisses the figures offered by the present writer with the remark that "they would

<sup>1</sup> Denis: This *Journal*, viii, p. 401, November, 1910.

<sup>2</sup> Benedict: *Ibid*, vi, p. 363, 1909.

tend to show that in the hands of its originator the new method is capable of giving accurate results for total sulphur in urines in a fraction of the time usually required for this determination." However true is this assertion, it may be noted that Schmidt<sup>1</sup>, in the same number of the journal containing Denis' paper, reports figures exactly parallel with those in my original paper, and states that similar results were obtained by other analysts in the same laboratory.

Denis' only criticism of the present writer's process is that spattering occurs during the ignition. Schmidt<sup>1</sup> reports that "any slight loss due to spattering, which may sometimes take place when oxidizing with copper nitrate, can be prevented by covering the evaporating dish with a watch glass." Instead of using this simple expedient, Denis proposes to modify the original method in two respects, viz., by altering the composition of the oxidizing solution somewhat, and by using 25 cc. of urine for each determination, instead of the 10 cc. called for in the unmodified process. The use of 25 cc. of urine instead of 10 cc., is, to the mind of the present writer unfortunate, since it requires a greater quantity of urine (a factor of importance sometimes) and makes the evaporation over twice as long as in the original process. It also introduces, according to my experience in using copper nitrate for oxidizing for total sulphur in urine, a serious possible source of error which Denis appears not to have considered. If results are to be accurate, the decomposition of the nitrate during the final heating must be absolutely complete. This can be secured with certainty only by heating every particle of the residue to redness for at least ten minutes. With small dishes this is easy of accomplishment. The use of larger evaporating dishes, and having a greater bulk of residue scattered over a larger surface makes the final decomposition much more uncertain. Where care is exercised and a good Bunsen flame is used this fact might not have been brought out in a comparatively short series of determinations, and so may have escaped Denis. During, however, the hundreds of determinations carried out by the original process at Chicago it was demonstrated beyond question that in the hands of general analysts the small dishes, and the smaller quantity of

<sup>1</sup> Schmidt: *This Journal*, viii, p. 423, November, 1910.



urine, are safer. It may be noted that in the original directions for the method the word *small* as describing the evaporating dish to be used is italicized, and its dimension is specifically stated, 7-8 centimeters. The above considerations furnish the explanation of these detailed directions upon an apparently minor point. From experience in this work it is believed by the writer that the original process will be found satisfactory, and it is to be questioned whether Denis' modification is so safe for general use.

In conclusion it may be pointed out that the finding by Schmidt that the copper nitrate method is not so satisfactory for urines containing albumin, is not a serious defect. Customarily it is not desired to estimate the protein sulphur along with the normal forms of urinary sulphur. Boiling and filtering samples of albuminous urine, prior to employing them in any total sulphur determination would seem desirable.

It is the hope of the writer, in connection with Dr. E. E. Gorsline of this laboratory and New York Hospital, to extend the copper nitrate method of ashing urine to sodium, potassium, magnesium, calcium, and phosphorus determinations.



# ON THE CONCEPTION AND DEFINITION OF THE TERM CATALYSOR.

BY ALONZO ENGLEBERT TAYLOR.

(Received for publication, December 5, 1910.)

In a recent paper Bradley<sup>1</sup> has discussed the differences in the actions of ferments as contrasted with those of catalysors. In common with many physiological chemists, Bradley attempts to demonstrate fundamental differences in the properties of the ferments and of the chemical (organic or inorganic) catalysors. In the opinion of the writer, there are no such fundamental distinctions between catalysors and ferments, and the latter ought to be considered only as a special class of catalysors, of labile constitution and operating in complex systems where the chemical opportunities for qualitative and quantitative deflections are numerous. The error of the point of view of Bradley lies not in the statements of what the ferments do, but in a definition of the properties of the catalysor so rigid as to exclude undoubted facts determined for them.

According to the older definition of "catalysor" of the Ostwald School, a catalysor was a substance that accelerated the velocity of an already existing reaction without altering the order of the reaction, the equilibrium in the system, without combination with either the substrate or the products of the reaction, and which in the end was to be found unchanged among the products of the reaction whose velocity it accelerated. With later work, it became clear that the best interpretation of the *modus operandi* lay in the theory of intermediary reactions; the action of the catalysor was held to lie either in the reduction of the number of intermediary stages in the auto-reaction; in the substitution of other intermediary stages of greater velocity than those in the auto-reaction; or in both. The entire scope of the function of the

<sup>1</sup> Bradley: this *Journal*, viii, p. 251, 1910.

catalysor was restricted to the reduction in the internal chemical resistance of the reaction; it could of course not alter the driving force of the reaction.

These older criteria of a catalysor cannot be held *in toto* to-day, since they are contradicted by experimental evidence. In the first place, van't Hoff<sup>1</sup> has pointed out that under certain circumstances it must be assumed that a positive catalysor may inaugurate a reaction in a system in a state of rest. With this aspect of the question we are not here concerned. Investigations have however revealed instances in which the catalysor combines with the substrate of the reaction, instances in which the catalysor combines with the products of the reaction, and instances in which the presence of the catalysor alters the order of the reaction. Since all of these must operate to alter the equilibria in the systems concerned, it follows that when a ferment alters the equilibrium of a system, it does nothing peculiar to ferments or unknown of catalysors. It may be remarked in passing that the rule of Schütz has been given its best explanation, experimental and mathematical, in the work of Arrhenius<sup>2</sup> who regards it as the expression of the law of mass action relating to the chemical combination between the ferment and the products of the reaction. Without attempting a search of the literature, one illustration of the instances above noted will be adduced.

MacIntosh<sup>3</sup> studied the acceleration of the reduction of hydrogen peroxide by colloidal silver. The catalysor was found to combine with a portion of the substrate to form a compound that was catalytically inactive. In other words, the mass of the substrate which, according to the law of mass action, is one of the factors in the equilibrium, was altered in the system containing the catalysor.

An exceedingly complex reaction, but one that probably illustrates both the combinations of the catalysor with the substrate and the products of a reaction, is the action of hydrochloric acid upon the formations of the isomers of cinchonine. Wegscheider<sup>4</sup>

<sup>1</sup> Van't Hoff: *Vorles. ü. theoret. ü. physikal. Chem.*, i, p. 206.

<sup>2</sup> Arrhenius: *Med. Nobelinstitut*, i, No. 9.

<sup>3</sup> MacIntosh: *Journ. of physical Chem.*, vi, p. 15.

<sup>4</sup> Wegscheider: *Zeitschr. f. physikal. Chem.*, xxxiv, p. 290; xxxv, p. 565.

has studied carefully the kinetics of this reaction, and has come to the following conclusion: The auto-reaction is an addition reaction, yielding HCl-cinchonine. The second reaction, the transformation reaction, consists in the formation of the isomer *a-i*-cinchonine, with which the HCl also forms an addition product. The hydrogen ion of the HCl can be shown not to be the catalysor; this is to be sought in the reaction of addition, which in some unclear way yields the accelerating influence. Since these reactions and combinations are held to operate in accordance with the law of mass action, it is clear that any variation in the concentration of hydrochloric acid must lead to a shifting in the equilibrium.

An illustration of the combination of the ferment with the products of the reaction is to be found in the action of colloidal silver on the hydrolysis of ethyl acetate. In itself an unsatisfactory catalysor for this reaction, the progression is made still more unsatisfactory by the fact that the silver combines with one of the products of the reaction, acetic acid.

The reaction:  $\text{H}_2\text{O}_2 + 2 \text{HI} = 2 \text{H}_2\text{O} + \text{I}_2$ , is a bimolecular reaction. When however, as shown by Brode,<sup>1</sup> this auto-reaction is accelerated with molybdic acid and iron sulphate, the velocity is simply a function of the concentration of hydrogen peroxide. In other words, a reaction of the bimolecular order has been converted into a reaction of the monomolecular order, with the consequent inevitable alteration in the equilibrium.

When catalytic reactions in organic systems and in particular with organic catalysors are studied, it will certainly be found that the Simon-pure catalysor will be often missed. The labilities of the systems are so marked and the possibilities in the system so numerous, that combinations between the catalysor and products will surely be often encountered. It is here as elsewhere; the definition of the ideal case will not often be found under complex experimental conditions. The law of conductivity holds only for infinite dilutions. Even the simplest formulation of the law of mass action,  $-\frac{dC_1}{dt} = kC_1 C_{11} \dots$  holds only for dilute solutions. And so the simplest and most ideal formulation of cata-

<sup>1</sup> Brode: *Zeitschr. f. physikal. Chem.*, xxxvii, p. 257.

lytic reaction can be expected to hold only for the simple conditions. As Spring has taught us, from the most strictly colloidal substance to the most strictly crystalloidal substance is a long line of gradual transition, with substances in every stage. And so the accelerators of auto-reactions constitute a long line, with the most stable inorganic catalysors at the one end, and the most unstable of organic ferments at the other end. A distinction between ferments on the one hand and catalysors on the other, on the ground that the ferments can alter the equilibrium of the system and catalysors do not, is incorrect for the simple reason that some catalysors do alter the equilibrium in the system precisely as ferments do.

# ON THE REFRACTIVE INDICES OF SOLUTIONS OF CERTAIN PROTEINS.

## IV. CASEIN IN ALCOHOL-WATER MIXTURES.

By T. BRAILSFORD ROBERTSON.

(From the Rudolph Spreckels<sup>1</sup> Physiological Laboratory of the University of California).

(Received for publication, November 22, 1910.)

I have previously shown<sup>1</sup> that if casein be dissolved in alkaline or acid aqueous solutions the change in the refractive index of the solvent is directly proportional to the concentration of the casein which is dissolved therein. The change in the refractive index of the aqueous solvent which is brought about by the addition of one gram of casein is, between 20° and 40°, independent of the temperature and equal to 0.00152.

I have also shown<sup>2</sup> that solutions of serum globulin can be obtained in alkaline alcohol or acetone-water mixtures and that the influence which the globulin exerts upon the refractive indices of these solvents varies considerably with their alcohol or acetone content.

With a view to further investigation of the influence of the nature of the solvent upon the power of dissolved protein to change its refractive index, I have determined the refractive indices of solutions of casein in alkaline alcohol-water mixtures of varying alcohol-content.

The casein which was employed in these experiments was prepared by further purification of Eimer and Amend's C. P. casein "Nach Hammarsten." The method of purification has been described in previous communications.<sup>3</sup>

<sup>1</sup> T. Brailsford Robertson: *Journ. of Physical Chem.*, iii, p. 469, 1909; *Journ. of Indust. and Engineering Chem.*, October, 1909.

<sup>2</sup> This *Journal*: viii, p. 441, 1910.

<sup>3</sup> T. Brailsford Robertson: *Journ. of Physical Chem.*, xiv, p. 528, 1910.

If 0.5 cc. of a .0125 N solution of potassium hydroxide, neutralised either to phenolphthalein or litmus by the addition of casein, be added to 10 cc., *i.e.*, to 20 volumes of 99.8 per cent alcohol (Kahlbaum's, C. P.) no precipitation of protein occurs, although the solution is very appreciably more opalescent than a solution of equal concentration in water containing no alcohol. Even if, instead of employing a solution of potassium caseinate in water, we employ a .0125 N solution of potassium hydroxide neutralised to phenolphthalein or to litmus by casein, in 75 per cent alcohol, adding 0.5 cc. of this to 10 volumes of alcohol still no precipitation of the casein occurs, although it is now dissolved, or forms a stable suspension in a 98.6 per cent solution of alcohol.

On progressively adding alcohol to solutions, alkaline or neutral, of caseinates of the alkalies no change occurs in the appearance of the solution until the concentration of alcohol attains a value lying between 60 per cent and 75 per cent, when a sudden and very marked increase in opalescence occurs; on further addition of alcohol no further change in the appearance of the solution can be observed.

A 12 per cent solution of casein in  $\frac{N}{10}$  potassium hydroxide was prepared. If the whole of the casein be introduced at once lumps are formed which are only broken up and dissolved with difficulty, hence the casein was introduced, while rapidly stirring, in four equal portions, waiting each time until the last portion was dissolved before adding another portion.

Twenty-five, 20, 15 or 10 cc. of this solution were carefully measured into a 100 cc. volumetric flask. To this was added 0, 5, 10 or 15 cc. of  $\frac{N}{10}$  potassium hydroxide, making the total volume of tenth-normal potassium hydroxide 25 cc. To this was then added 0, 25 or 50 cc. of 99.8 per cent alcohol and the total volume of the mixture was made up to 100 cc. In preparing the solutions in 75 per cent alcohol, to the 25 cc. of casein solution in  $\frac{N}{10}$  potassium hydroxide 99.8 per cent alcohol was added until the volume of the mixture was 100 cc.

The measurements of the refractive indices of the solutions containing no alcohol were carried out, not only for purposes of comparison, but also in order to afford a check on the concentration of solutions made up in this way.



All of the determinations were made at 25° C. in a Pulfrich refractometer reading the angle of total reflection to within 1', a sodium flame being employed as a source of light.

The following were the results obtained. The values headed "a" are calculated from the formula,  $n - n_1 = a \times c$  where  $n$  is the refractive index of the solution,  $n_1$  that of the solvent and  $c$  is the percentage of casein in the solution.<sup>1</sup>

TABLE I.

.025 N KOH: Alcohol, 0 per cent.  $n_1 = 1.33170$

$c$ = Grams Casein in 100 cc. of Solution	$n$ = Refractive Index of Solution	$a = \frac{n - n_1}{c}$
3.0	1.33615	.00148
2.4	1.33529	.00150
1.8	1.33442	.00151

TABLE II.

.025 N KOH: Alcohol, 25 per cent.  $n_1 = 1.34527$

$c$ = Grams Casein in 100 cc. of Solution	$n$ = Refractive Index of Solution	$a = \frac{n - n_1}{c}$
3 0	1.35000	.00158
2 4	1.34906	.00158
1 8	1.34813	.00159
1.2	1.34711	.00153

TABLE III.

.025 N KOH: Alcohol, 50 per cent.  $n_1 = 1.35650$

$c$ = Grams Casein in 100 cc. of Solution	$n$ = Refractive Index of Solution	$a = \frac{n - n_1}{c}$
3.0	1.36100	.00150
2.4	1.36011	.00150
1.8	1.35914	.00147
1.2	1.35826	.00147

<sup>1</sup> Cf. previous papers of this series in this *Journal*.

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TABLE IV.

.025 N KOH: Alcohol, 75 per cent.  $n_1 = 1.36226$

$c$ = Grams Casein in 100 cc. of Solution	$n$ = Refractive Index of Solution	$a = \frac{n - n_1}{c}$
2.4*	1.36522	.00123
1.8	1.36450	.00124
1.2	1.36378	.00127

\* The 3 per cent solution in 75 per cent alcohol was too opalescent to enable a satisfactory reading to be obtained in the refractometer.

It is evident that the addition of 25 or 50 per cent of alcohol to the solvent causes very little change in the influence which casein exerts upon the refractive indices of its solutions, but that concurrently with the molecular change which renders solutions of casein in 75 per cent alcohol so markedly opalescent, a marked diminution occurs in the power of dissolved casein to alter the refractive index of the solvent.

In order to obtain a satisfactory estimate of the probable degree of accuracy of these determinations it is necessary to recollect that each determination of the angle of total reflection is liable to an error of  $1'$ . An error of  $1'$  in the determination of the angle of total reflection corresponds to an error of from .00008 to 0.00010 in the determination of the refractive index. We will assume the higher value. Now it is obvious that since the absolute error in each determination of  $n - n_1$  is the same, the error in the determination of  $a$  must be less in proportion to the magnitude of  $c$ . In order to assign to each determination its due weight in the estimation of the mean value of  $a$  for any solvent we must therefore add together all of the observed values of  $n - n_1$  and divide this sum by the sum of the concentrations employed. Proceeding in this way we obtain the following values of  $a$ .

$$\text{Alcohol, 0 per cent; } \frac{\Sigma (n - n_1)}{\Sigma c} = \frac{.01076 \pm .00030}{7.2} = .00149 \pm .00004$$

$$\text{Alcohol, 25 per cent; } \frac{\Sigma (n - n_1)}{\Sigma c} = \frac{.01322 \pm .00040}{8.4} = .00157 \pm .00005$$

$$\text{Alcohol, 50 per cent; } \frac{\Sigma (n - n_1)}{\Sigma c} = \frac{.01251 \pm .00040}{8.4} = .00149 \pm .00005$$

$$\text{Alcohol, 75 per cent; } \frac{\Sigma (n - n_1)}{\Sigma c} = \frac{.00672 \pm .00030}{5.4} = .00125 \pm .00006$$

The first three values are thus seen to be, within the experimental error, identical; but the value of  $a$  in 75 per cent alcohol is very much less.

#### CONCLUSIONS.

1. The value of  $a$  in the equation  $\frac{n - n_1}{a} = c$  where  $n$  is the refractive index of the solution of the protein,  $n_1$  that of the solvent and  $c$  is the percentage concentration of the protein has been determined for casein in alcohol-water mixtures of varying alcohol-content.

2. The following were the values obtained:

In Alcohol = 0 per cent:  $a = .00149 \pm .00004$

In Alcohol = 25 per cent:  $a = .00157 \pm .00005$

In Alcohol = 50 per cent:  $a = .00149 \pm .00005$

In Alcohol = 75 per cent:  $a = .00125 \pm .00006$



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